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Award Number: W81XWH-09-1-0039

TITLE: T Cell Gene Therapy to Eradicate Disseminated Breast Cancers

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REPORT DATE: May 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE May 2012		2. REPORT TYPE Annual		3. DATES COVERED 1 May 2011 – 30 April 2012	
4. TITLE AND SUBTITLE  T Cell Gene Therapy to Eradicate Disseminated Breast Cancers				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-09-1-0039	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)  Richard P. Junghans, M.D., Ph.D.  E-Mail: Rjunghans@rwmc.org				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  Roger Williams Medical Center Providence, RI 02908				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Background: There is no cure for metastatic breast cancer, which kills 40,000 American women (and 500 men) each year: all presently available treatments are palliative. Gene therapy techniques are used to introduce chimeric immunoglobulin-T cell receptors (IgTCR) into autologous patient T cells to create "designer T cells" that redirect the T cell immune system in a new type of immuno-gene therapy against breast cancer. Designer T cells have been created against the carcinoembryonic antigen (CEA) that is prominently present on many metastatic breast tumors (30-60%). This exceeds the fraction that are Her2/neu overexpressing (20-25%), making CEA an even better immune target for attacking breast cancer. Building on a prior study of CEA designer T cells in breast and colon cancer, "2nd generation" designer T cells were created by incorporating into the IgTCR a CD28 co-stimulation cassette that was shown to oppose activation-induced cell death (AICD) of the T cells after tumor contact. This advanced generation modification leads to improved designer T cell survival and improved anti-tumor potency in preclinical models. Although the 2nd generation designer T cells produce interleukin 2 (IL2) growth factor on contact with tumor, interleukin 2 (IL2) supplementation is anticipated still to be required for optimal clinical therapeutic effect. However, the CBER/FDA has mandated a Phase I					
15. SUBJECT TERMS Breast Cancer, Designer t cells, co stimulation					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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## Annual Report

### INTRODUCTION

The following are the study Aims. We report progress on three Aims: 1, 2 and 3 (marked by \*).

#### I. Clinical (existing agent)

\*Aim 1. Test 2<sup>nd</sup> gen designer T cells for OBD and efficacy in metastatic breast cancer

\*Aim 2. Test ancillary procedures for improved persistence and activity of infused cells

#### II. Pre-clinical: Advanced Research & Development

\*Aim 3. Devise and preclinically test 3<sup>rd</sup> generation CARs

\*3.1. To create and test other CAR designs with alternative co-stimulatory domains

\*3.2. To create and test CAR to avoid need for IL2 supplement in vivo to sustain T cell survival

#### III. Clinical (new agent)

Aim 4. Test safety and efficacy of 3<sup>rd</sup> gen designer T cells

### BODY

#### **Aim 1. To test 2<sup>nd</sup> gen designer T cells in metastatic breast cancer**

##### **A. Background**

This Aim applies a randomization of 12 subjects to –IL2 or +IL2 arms at the maximum tolerated dose (MTD) or maximum practical dose (MPD) of designer T cells under a Phase Ib design. This will test the optimal biologic dose (OBD) in terms of the benefit of IL2 to T cell persistence and activity in vivo.

There were three dose levels in the original Phase Ia:  $10^9$ ,  $10^{10}$  and  $10^{11}$  T cells. During this report period, we treated our final patient on the  $10^{10}$  dose (level 2), completing the first two cohorts. In the first two cohorts, all patients had good tolerance of the designer T cells. One patient had stable disease without other effective treatment for 12+ months. One patient had a minor response to treatment, with shrinkage of brain and lung mets, but with resurgence of disease in subsequent months. This is a situation in which we postulated that addition of IL2 will allow deeper and more prolonged response. Our prior animal studies have suggested that even 2<sup>nd</sup> gen dTc need IL2 in vivo for eradication of established tumors.

##### **B1. Dose preparation**

There are two elements of dose preparation: (1) T cell modification (creating designer T cells), and (2) T cell expansion (to meet the dose size requirements). During the recent period, we made significant advances in both areas.

##### **(1) T cell modification**

During the Phase Ia portion of the study, we confronted modest dTc modification efficiencies (10-20%) that got worse with the mid-dose level, where more cells had to be modified. This was in large part due to poor vector titer from an error at the National Gene Vector Lab that prepared the vector under contract to us. Another vector we had made against a different tumor antigen

had modifications routinely in the 50-60% range. During the prior period, we addressed this problem by two means:

**(a) *Retronectin-enhanced transduction.***

As previously described, results showed a 50-100% increase in transduction efficiency. Requisite approvals were obtained for use of clinical grade retronectin in our trial. During this period, we applied this in the final patient to complete the Phase Ia, second cohort of designer T cell treatment and then in further doses (below) with improved Td efficiency of 25-30% was obtained versus levels of 5-15% previously.

**(b) *New vector supernatants.***

As mentioned above, the NGVL initially mis-prepared our vector: they manufactured the virus at the wrong temperature (37C), which we have shown greatly reduces titer versus our standard collection temperature (32C). During year 2, the NGVL reprepared a new batch of vector at a reduced cost \$50,000 to the grant (normally \$150-200,000). The titer is 50% better than the prior batch. The batch underwent safety testing and was released in the Q6 period for human use. This material was preserved until the first lower titer lot was exhausted, which occurred in this current year (below).

**(c) *Vector dilutions.***

We previously showed that vector dilution can be performed without loss of transduction efficiency, down to 1:3. This is due to inhibitors in the medium that the VPCs produce. When the inhibitor diluted out, the transductions increase, until the virus level declines too greatly.

Since this time, we have done more extensive dilution tests that show adequate titers with 5- to 10-fold dilutions. This will allow conservation of the vector supernatants, which are very expensive. During these experiments, we also confirmed the benefit of sequential transductions with diluted supernatants. With a single Td, there was a two-fold difference in Td efficiency between 10-20% supernatants. With three Td, the difference was only about 20%. We also confirmed that there were only modest differences between the different harvests over the lifetime of the vector producer cell (VPC) culture.

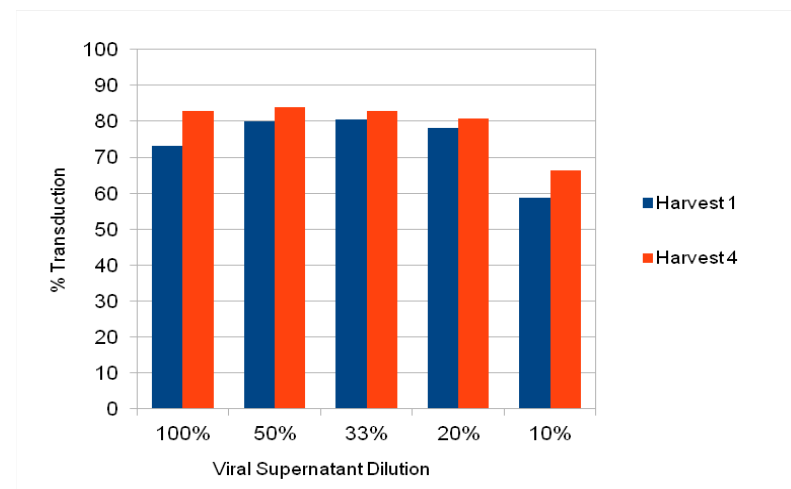


Fig. 1. Supernatant dilutions allow excellent transduction (Td) while conserving supernatant. T cells are modified with 3x Td plus retronectin (RN).

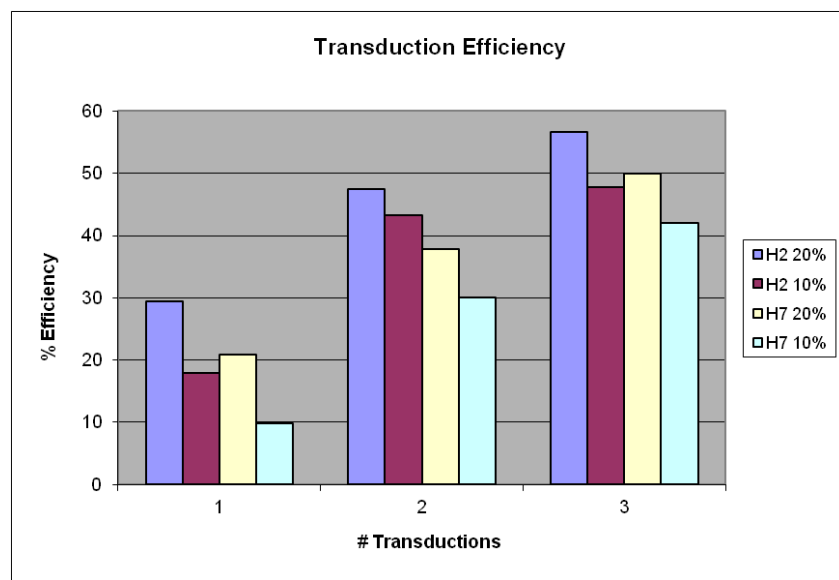


Fig. 2. Effect of number of transductions on the % modified with different dilutions of two supernatant harvests. Three Td appears to make 10% and 20% supernatants approximately equal, whereas a single Td has 10% supe significantly less effective than 20% supe.

## **(2) T cell expansion**

### ***(a) Background***

For the Phase Ib portion under DOD funding, we needed to go up one log in our expansions to achieve dose. We were generally successful with the  $10^9$  and  $10^{10}$  doses. But the  $10^{11}$  dose level presented significant challenges. For example, patient #1 (CS) at the  $10^{11}$  target dose level had the following transduction and expansion data, starting with  $10^9$  cells, our maximum for Td.

CS Harvest 1:            4 TD's performed (300 ml vector/TD) 1200 ml vector used in run  
                               Total Cells: 12.7E9                            % Modified = 51.4 %

CS Harvest 2:            4 TD's performed (300 ml vector/TD) 1200 ml vector used in run  
                               Total Cells: 11.1E9                            % Modified = 42.3 %

All transduction s (Td) had good efficiencies (42-51%) while employing the old vector with RN. However, as reported previously, this patient's T cell expansions were not optimal. Despite two expansions and pooling them for her dose, each dose only increased about 10-fold from the activated T cells to the expansion. This was adequate for the preceding doses of  $10^{10}$  cells, but falls far short of the target dose of  $10^{11}$ .

### ***(b) Strategies for improving dTc expansions.***

Prior tests showed that the amount of OKT3 antibody in the activation (30-60 ng/ml) had no impact. However, high doses of IL2 did have an impact, seen in the Figure, described in the prior year's report.

With these data, we changed the manufacturing SOP to incorporate high IL2, effective with patient #2 on the Phase Ib. This is a significant additional cost in terms of IL2, but it is completely justified by these results.

Since the prior report, we have noted that some individuals have a better response to the high dose IL2 for their expansion kinetics than do others. Our working hypothesis is that patients receiving more or different kinds of chemotherapy may damage the patient T cells with worse responsiveness of their T cells to activation stimuli. This will require a prospective evaluation as we gain more experience with patient cell modifications.

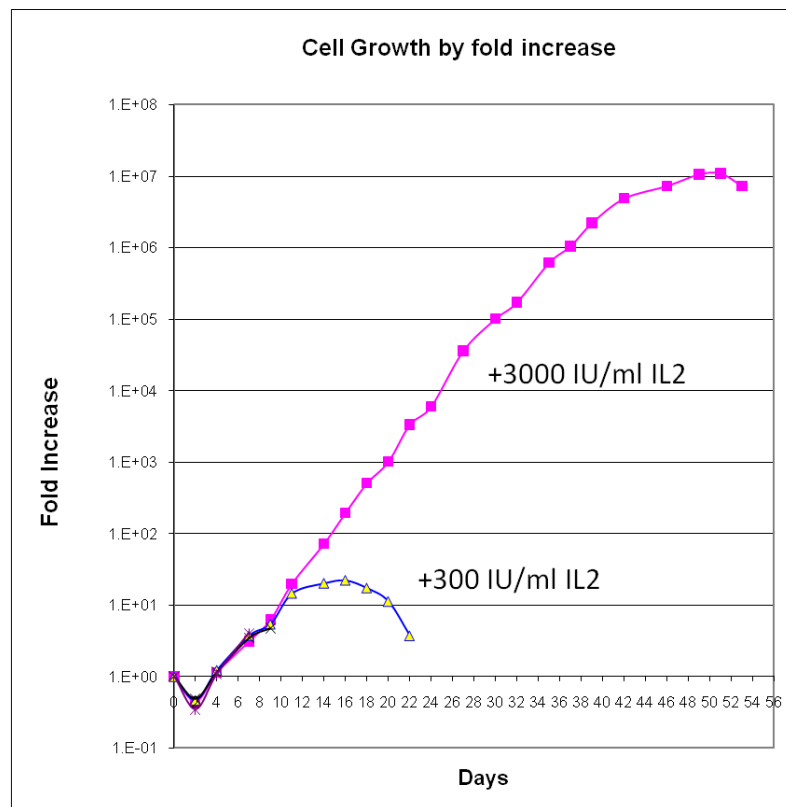


Figure 3. High IL2 extends proliferation of activated T cells.

**(c) Application to Prometheus to permit high-dose IL2 for expansions.**

These data were shared with Prometheus (licensed by Novartis to manage Proleukin [IL2]). In the past year, after preparation of a proposal and submission for their internal review, the company consented to continue its support of the trial with IL2 under this expanded requirement. See Appendix.

**(d) Patient dose**

This method was applied in the instance of patient #2 (VS). She had a much improved expansion, ultimately harvesting after a 50-fold growth,  $>5 \times 10^{10}$  cells, which was 50% of target, in a single expansion.

VS Harvest 1: 3 TD's performed (150 ml vector/TD for TD 1 and 2)  
 (200 ml vector used for TD 3) 500 ml vector used in run  
 Total Cells: 53.0E9                      % Modified = 42.9 %

Although this was a substantial improvement over the dose expansion with Patient #1, the net expansion at 32 days (50-fold) was still far less than that of the control preclinical tests we did with normal random donors (1000-fold). This difference could be due to the effect of multiple prior chemotherapies to damage the patient T cells; to determine this, we will need to see how

different patient donors do in parallel tests with normal donors. This will be part of our plan in the coming year.

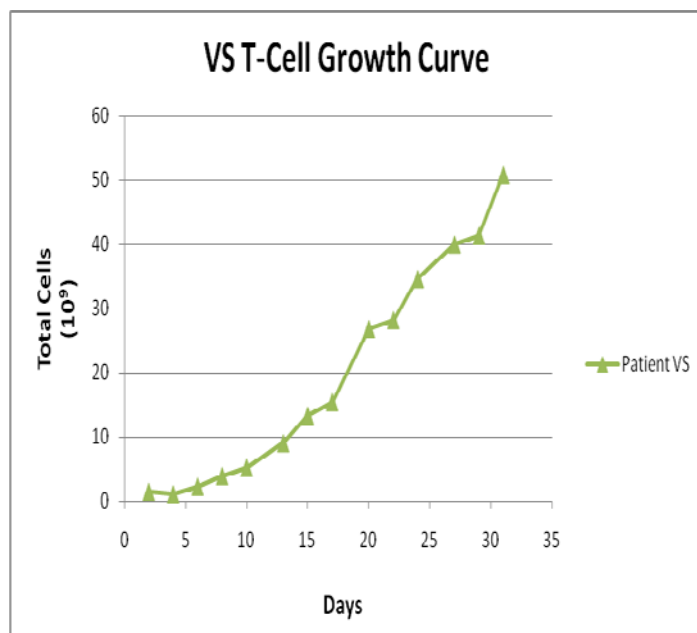


Fig. 4. Patient #2 (VS) growth curve with high IL2 (3000 IU/ml)

## B2. New Manufacturing Procedures:

### Reviewing ATC expansion methods for GMP manufacturing

Our prior Fenwal equipment used for cell expansions and harvesting has been discontinued for support by Baxter, with disposables no longer being available. This has necessitated adoption of new procedures. We are presently reviewing new expansion procedures. One of the lab senior staff members (Q Ma) spent the day at NIH in Dr Steven Rosenberg's lab to review their procedures for T cell expansions. We plan in the next two months to create and validate the new SOPs with the aim of having a more reliable expansion to high doses with new equipment available (Greiner flasks, wave bioreactors). We are attaching documentation for each of these.

**Gas-Permeable Cell Culture Bags** Flexible, gas-permeable pre-sterilized disposable cell-culture bags are commercially available for ATC production. The bags feature attached ports, and tubing with roller clamps, which are used for inoculation of cells and media, sampling during production, and harvest. The bag is inoculated with cells and media, placed in a CO<sub>2</sub> incubator, and generally handled as a batch culture. Cell culture bags have some other advantages over dishes or flasks: the bags are completely enclosed systems, which reduces the opportunity for microbial contamination, and they may be placed flat on the incubator floor or hung on a stand, potentially reducing required incubator space. However, the permeability of cell culture bag to CO<sub>2</sub> and oxygen is not enough to support ATC to grow to high cell densities (can only go to up to 2x10<sup>6</sup>/ml). For clinical protocols designed for cell doses greater than 1x10<sup>10</sup> T cells, it requires many bags and space, which exponentially increase the cost and labor.

**G-Rex flasks.** G-Rex flask is the most efficient gas permeable cell culture device on the market. The G-Rex advantage is obtained by a geometric design that allows cells to reside upon a gas

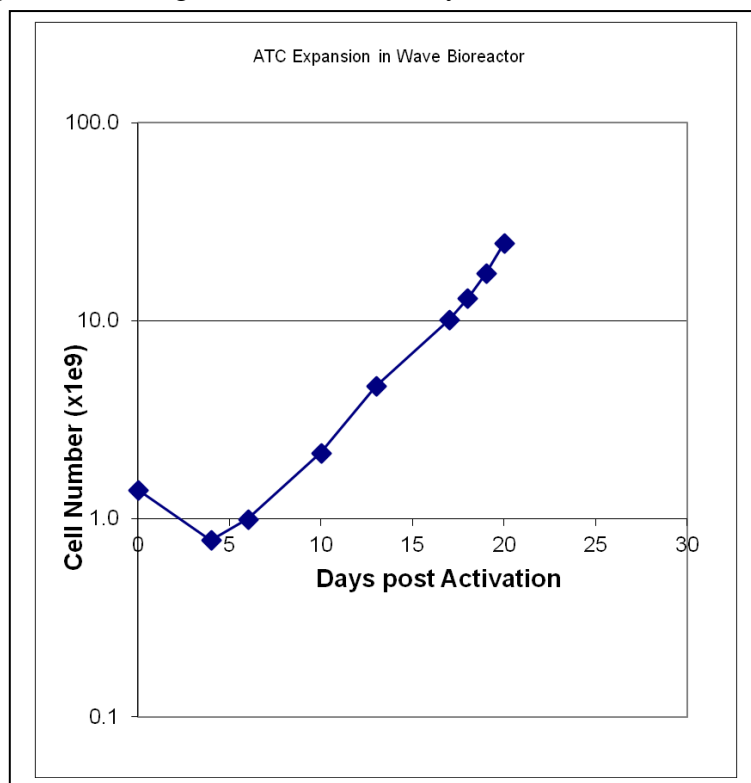


permeable membrane and allows medium to reside at a height beyond that of any commercially available device. Cells experience virtually unlimited access to oxygen and nutrients. This patent pending approach results in the most space efficient and labor efficient cultureware on the market. Cells have virtually unlimited access to oxygen on demand. By allowing the cells to gravitate to the gas permeable membrane, they are in an undisturbed state and in optimal proximity of ambient incubator gas. As cells consume oxygen, a concentration gradient forms across the membrane, and oxygen from the ambient gas of the incubator automatically moves through the gas permeable membrane of the G-Rex to replace the oxygen consumed by the cells. Cell densities can go up to  $10 \times 10^6$  cells/ml. Compared to cell culture bag, G-Rex can save space and medium.

**Wave Bioreactors** For doses greater than  $1 \times 10^{10}$  T cells, the wave bioreactor is a suitable option. The wave bioreactor is a flexible, plastic pre-sterilized, disposable cell culture bag. Cell densities can up to  $10 \times 10^6$  cells/ml. The volume can from 2L to 500L. A single bag can make one batch. Compared to other methods, the labor and space requirements are optimized. The user partially fills the bag with culture media and cells, inflates it to rigidity, and maintains it on a rocking platform in a CO2 incubator. The headspace in the bag is continuously aerated. These bags are not gas-permeable; the wave-induced agitation generated by the rocking mechanism effects oxygen transfer and mixing. The wave action increases the air-liquid surface area for oxygen transfer, mixes the fluid in the bioreactor, and suspends the cells with low shear. The bag has an inlet air filter and exhaust air filter, a needle-less syringe fitting for sampling from the bag, and a tubing connector for additions to, and harvests from, the bag. A laminar flow hood is not required for adding to, nor sampling from, the bag. The bioreactor may be handled as a batch or fed-batch culture system. In fed-batch mode, the volume of media may be increased gradually as cell density increases.

The wave bioreactor will be used for the high dose with the current breast cancer patients. It is new technology for us. The data from the current test expansion are shown in this Figure.

Figure 5. Initial growth curve in wave bioreactor. The goal is  $100 \times 10^9$  T cells, starting from  $\sim 1 \times 10^9$ , or a 100-fold expansion. In this present test, two further doublings will achieve this goal. This experiment is on-going at the time of this filing.



We are presently planning to replace our current cGMP manufacturing technology from cell culture bags to G-Rex Flasks for manufacturing small to medium doses (up to  $10^{10}$ ) and Bioreactor for large doses ( $10^{11}$ ).

### **B3. Facility Re-organization**

With departure of the former manager of the GMP, we have instituted a new staffing arrangement, with retraining and certification. This effort has involved a review and editing of all SOPs as well as creation of new ones. A major time effort has been expended so far, but will soon be completed.

Since the last report, we initiated a second phase re-organization of the cell therapy facility, with recruitment of a new facility director, who arrived on May 1. We also initiated new construction for the QC component of the lab, and added UV lamps for the clean room.

Due to activities under the current phase of re-organization, dose preparations have been on hold since the last report. We are anticipating resumption of patient dose preparation in September 2012, with enrollments planned to follow at a rapid pace.

### **C. Patient Enrollments**

#### **(1) Patient recruitment**

In an earlier report, we indicated that Dr Susan Love/Avon Army of Women (LAAW) had reviewed and approved our proposal for patient recruitments. An email “blast” was sent by LAAW in June, 2011. This generated a large number of inquiries, and these are continuing at a reasonable pace.

The PI has also continued to give seminars in public forums to enhance physician awareness and patient referrals. During this period, the PI presented on June 15 at Lahey Clinic (Burlington MA) and June 17 at Rhode Island Hospital (Providence). The PI’s abstract was selected for oral presentation at the 2011 Era of Hope meeting in August in Orlando, and he presented there. The PI presented a seminar at the Dana Farber Breast Cancer section on November 9, with feedback of their interest to refer patients to the study. One of the fellows at this conference approached me to prepare a joint review on immunotherapy for breast cancer.

Since February, 2012, we have had 6 patient inquiries. Of the 6 patient inquiries one has had consent form reviewing but not signing.

Overall, since opening the study to patient enrollments under Army of Women one year ago, we have had inquiries from 62 patients (61 F, 1 M) from all across the country, as well as 19 inquiries from family members that never led to patient contact. Of the 62 patient contacts, 48 are coded for enrollment status. Among the 48 coded for enrollment, there were 8 enrollments (CF signing), 6 with doses prepared plus one with a dose near production (leukopheresis product collected, but T cell transduction and expansion delayed). One enrolled patient had no dose prepared because of disease progression. To date, two patients were treated. There is also one patient who had a dose in storage for use at a later date; patient then had disease progression then loss to follow-up. We expect the patient with the dose in production will be treated, as stated above. This would give a ratio of 8/62 proceeding to enroll after contact, and if treated as planned 3/8 getting treated after enrolling.

We attempted to analyze the low ratio of enrollments and progress to treatment. A table is attached that documents results of our patient recruitment activities with an assessment of the reasons for not enrolling, or not treating if enrolled. This table is not fully completed, but some provisional conclusions can be offered.

We have 54 patients from a group of 62, who were not enrolled in our clinical trial due to different reasons. 27 patients were on other treatments but looking for information and still could decide to enroll, a further 13 patients were indecisive and 5 patients were totally not interested in the trial. 6 patients declined due to the financial or logistic reasons. There were 3 patients with advanced disease and poor performance status whom we judged ineligible for the trial.

## **(2) Patient enrollment**

At the time of the last report, we had enrolled and treated the first patient on the Phase Ib under DOD support, and prepared a dose for patient #2 who unfortunately had rapid disease progression and could not be treated.

The Phase Ib has a randomization to -IL2/+IL2, with target dose of  $1 \times 10^{11}$  cells, or maximum practical dose (MPD).

The patients who enrolled are described in the following.

Patient #1 randomized to +IL2. She had a dose prepared that was  $0.2 \times 10^{11}$  T cells, under target, for reasons discussed previously. She had side effects attributed to IL2, with respiratory difficulty and a brief hospitalization. IL2 was discontinued after two days. During the 2 weeks following dTc administration, her tumor markers underwent a 20-25% decline, then resumption of their upward course after this period. It is possible that with full  $10^{11}$  dose of dTc and IL2 as planned, she could have had a sustained and much deeper tumor suppression.

Patient #2 randomized to +IL2. Her dose preparation yielded an improved level of  $0.5 \times 10^{11}$  cells under new manufacturing procedures. Unfortunately, this patient's clinical condition deteriorated due to cancer progression and she was excluded from treatment on the day of her scheduled infusion. (Drop-out)

Patient #3 randomized to +IL2. She enrolled on June 10, 2011. She underwent leukopheresis and dTc preparation. She unfortunately had a rapid decline in her medical condition and was cancelled for treatment. (Drop-out)

Patients #4, #5 and #6 enrolled on 9/28, 9/30 and 10/28/2011. Patients 4 & 5 had doses prepared but experienced rapid clinical deteriorations and were unable to be treated. (Drop-outs) Patient #6 had her dose prepared and stored since the prior report. She has bone-only disease, which is a more stable setting for breast cancer. We had planned for her dTc infusion in January. However, she developed soft tissue disease (pleural) that her oncologist wanted to treat. She is presently on chemotherapy, and doing well reportedly. We are in touch with the primary oncologist. It is likely she will be treated in the next month.

Patient #7 enrolled on 1/9/12. Her cells were transduced and expanded. She randomized to -IL2. She was infused on 3/5/12 (treated patient #2).

Patient #8 was enrolled on 2/10/12, with pheresis collection of T cells on 2/13/12. Cells were cryopreserved for later dose preparation, following facility reorganization and new SOPs.

The facility renovation and re-organization (below) has placed a hold on patient dose preparation and patient treatments. We expect to resume at a faster pace in September, 2012.

#### **D. Pharmacokinetics by PCR (detection of dTc in blood)**

Assays are already in place to follow DTC by flow cytometry, but this method is limited to detection when 1% or more of circulating cells. We have developed a PCR based approach that can detect at a 0.01% level. Because of homologies with endogenous elements in the human genome, we have had to adjust the primers to more selective domains. We learned there is interference of the assay from heparin in the cell collection tubes, and have now switched to sodium citrate as anti-clotting agent. During this recent year, we developed a new set of primers that now avoid interference from endogenous elements, and citrated samples have proven effective to work with. This improvement will be applied to the breast cancer patient samples.

##### **(1) Background.**

After significant troubleshooting, this assay has been established, and it is in use. Primer combinations have been tested spanning various sites in the 5' LTR of the MFG retroviral vector and specific for the MN14 antibody domain spanning the GS linker region. All primers displayed positive signal amplification in non-transduced T cells indicating background endogenous LTR and antibody amplification. Several primers spanning the chimeric CD28 and CD3 zeta domain were tested and found suitable for patient Q-PCR.

##### **(2) PCR Method**

###### Q-PCR Pharmacokinetics Protocol:

5 mL patient whole blood (WB) sample was collected using heparin\* or citrate coated BD vacutainer tubes (BD Biosciences). Genomic DNA was isolated from 200 uL sample using the AxyPrep blood miniprep kit (Axygen Biosciences) and eluted in 100 uL TE buffer. Real-time PCR was performed using the BioRad CFX96 PCR detection system (BioRad). Reactions contained 11 uL eluted sample, 14 uL Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) and 0.75 uL each primer at 10 uM. Primers were designed using Primer-Select (DNASTar) specific for CARs anti-PSMA (5-aggctgaggattgggagtt-3/5-agacgctccaggcttacta-3, 182-bp spanning the SD38 GS linker) and anti-CEA (5-gcaagcattaccagccctat-3/5-gttctggccctgctgta-3, 91-bp spanning the chimeric CD28-CD3z region) and albumin to quantitate absolute white blood cell (WBC) numbers (5-accatgcttttcagctctgg-3/5-tctgcatggaaggtgaatgt-3, 81-bp). Amplifications were at 95C for 10 min, 40 cycles at 95C for 15 s, 60C for 20 s and 72C for 20 s. Fluorescence data were acquired at the 72C extension phase. Product specificity was confirmed by melt curve analysis and gel electrophoresis. Absolute CAR copies and WBC numbers were calculated from plasmid standard curves and expressed relative to the baseline pre-screen (PS) collection point.

\*Heparin collection tubes contain heparin, a polymer of sulfated glycosaminoglycan carbohydrates which binds DNA and inhibits PCR by occupying polymerase binding sites. To remove heparin, 75 ul of sample was treated with 15 uL of Heparinase I Flavobacterium heparinum (Sigma) for 2 h at 37C. Heparinase I was dissolved at 1 mg per mL in 20 mM Tris-

HCl pH 7.5, 50 mM NaCl, 4 mM CaCl<sub>2</sub> and 0.01% BSA. 11 uL of heparin treated DNA was used for Q-PCR.

Notably, because of interference of heparin in the PCR reaction, we found it necessary to switch to citrated tubes instead, thus avoiding heparinase steps. This was successful. Citrated samples provided more reliable data. Moreover, assays were most reliable on whole blood rather than ficoll fractionated cells to collect PBMC.

### **(3) Patient data.**

#### **a. Patient analyses**

Samples from patient #1 were analyzed, showing a rapid clearance of CAR+ cells from the circulation. CAR+ cells expressed as percent of total circulating WBC. For this dose of dTc administered (2e10) and fraction modified (50%), the total CAR+ cells in blood would be about 40% if they remained in circulation. The first sample 15 min after completing the infusion is only 5%, and declines quickly thereafter. These are activated T cells that are expected to marginate on vascular endothelium and also to extravasate and enter tissues. The peak at 1 week follows the discontinuation of IL2 on day 3; it may or may not be real. More patients' samples will need to be analyzed to develop a clear pattern.

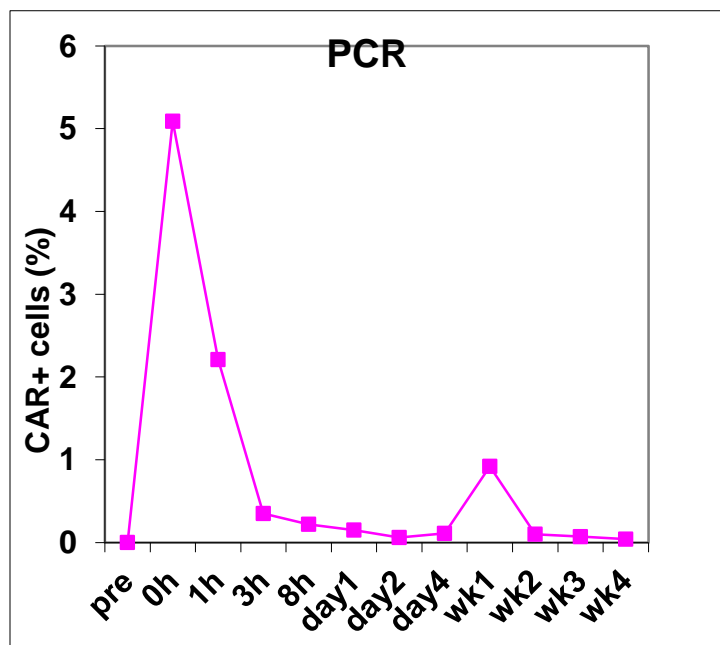


Figure 6. PK analysis by PCR of patient blood samples.

#### **b. Comparison of infusion versus engraftment method for dTc administration**

We recently completed analysis of dTc PK from patients in a study with dTc engraftment post chemotherapy lymphopenic conditioning. A comparison of patients on the two protocols shows graphically the impact of dTc infusion after conditioning in comparison with simple infusion.

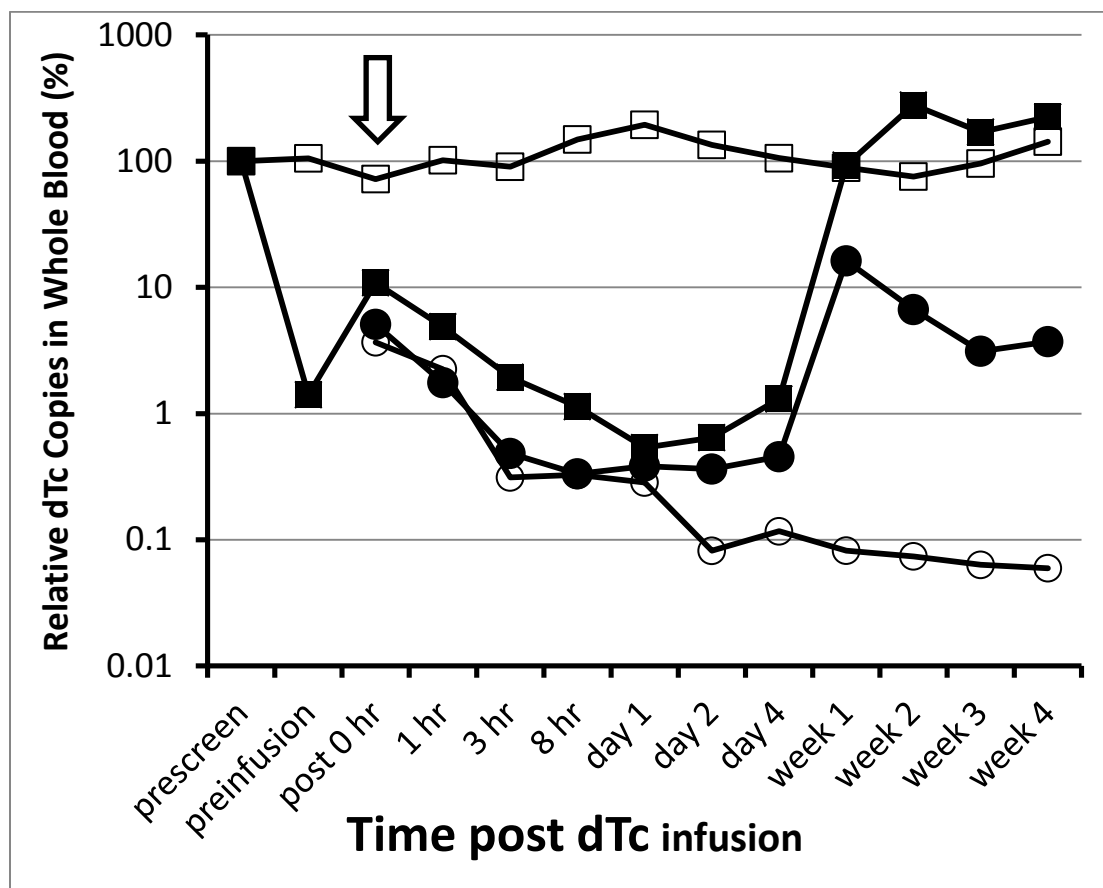


Fig. 7. Comparison of PK with straight infusion versus engraftment post lymphodepletion. The open symbols are from the breast ca patient with dTc infusion (patient #1, CS) without chemotherapy. Squares indicate total circulating WBC and circles represent dTc. Closed symbols are from the prostate ca patient with the dTc after chemotherapy.

In the first (breast) patient, without chemotherapy, the WBC is relatively constant through the course, as expected. In contrast, the infused dTc go down in a continuously declining fashion, beginning 4% of WBC and down to 0.06% by one month, for a near 60-fold decline.

In the second (prostate) patient, the WBC is low, after chemotherapy, down to 1% of starting, then increases momentarily because of dTc infusion, followed by another decline and then WBC finally recovers to normal levels by week 2 as the bone marrow recovers. Initially, the dTc started at about 4% of baseline WBC (like the breast ca patient), declined identically with the simple infusion thereafter, but then begin to pull away on day 2, jump up on day 7 and 14, with some modest decline afterwards. The dTc peaked at 15% by week 1 and then plateaued at 3-4%, for a near two-log gain in circulating dTc at one month by the engraftment procedure.

This analysis is important for guiding the subsequent test of engrafted anti-CEA dTc in our breast cancer study. This engraftment method will be undertaken when we have shown safety of the dTc at the highest dose in the context of +IL2 support for the dTc in vivo. This is under future Aim 2.2, to apply non-myeloablative conditioning with dTc therapy.

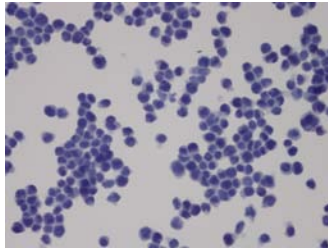
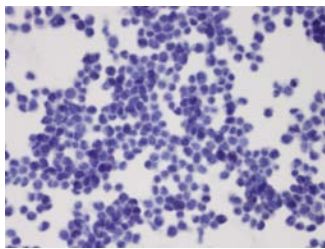
## **E. Staining for detection of dTc in tumor tissues**

One of the important measures of the intervention is to assess the trafficking and persistence/expansion of the dTc in vivo into tumor tissues. Initial tests confirmed the detection in frozen tissue but loss of detection in fixed. We have invested more efforts to determine if the CAR of the CAR-expressing T cells can be detected in fixed embedded tissues. For this, we recruited an able technician, Fang Xiong, who is experienced with IHC and IF.

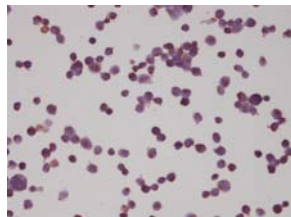
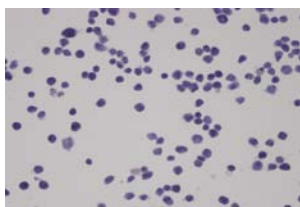
### **a. Tested WI2 antibody in formalin fixed cells using different antigen retrieval method**

**Results: good positive staining in designer T-cell, not in negative control slides and jurkat cells**

We compared heat induced epitope retrieval (HIER) and enzyme induced epitope retrieval (PIER) methods on our tissue samples. Heat induced epitope retrieval (we use the citrate-based solution, pH 6.0) can get the positive results. Without antigen retrieval, WI2 has no positive staining.



Jurkat cells. WI2 (1:25) staining. Chromagen: AEC. Nuclei: hematoxylin. formallin fixed. Left: negative control, Right: Jurkat cell

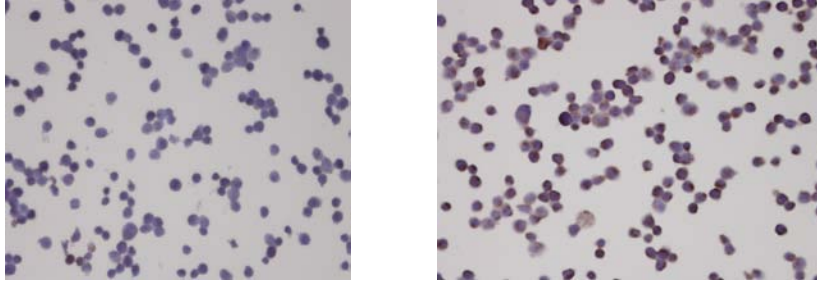


Designer T-cells. WI2 (1:25) staining. Chromagen: AEC. Nuclei: hematoxylin. formallin fixed. Left: negative control, Right: Designer T-cell

### **b. Tested CEA-Fc antibody in formalin fixed cells**

**Results: good positive staining in designer T-cell, not in negative control slides**

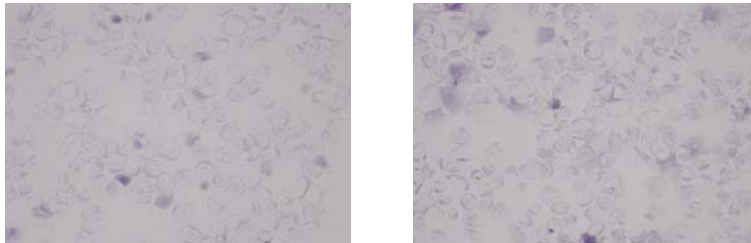
Jurkat cells. CEA-Fc (1:150) staining. Chromagen: DAB. formalin fixed. Left: negative control, Right: Jurkat cell (still need to test, no image so far)



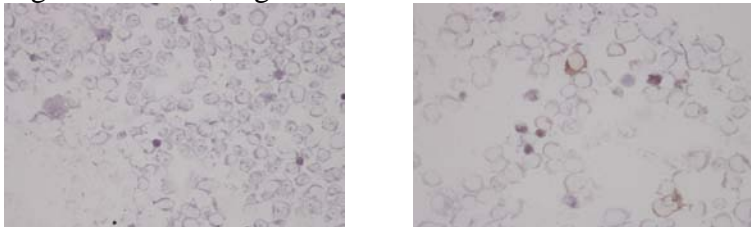
Designer T-cells, CEA-Fc (1:150) staining. Chromagen: DAB. Nuclei: hematoxylin. formalin fixed. Left: negative control, Right: Designer T-cell

### c. Tested CEA-Fc antibody in acetone fixed cells

**Results: a little positive staining in designer T-cell, not in control slides. Still need to work on the sensitivity of the staining.**



Jurkat cells. CEA-Fc (1:250), chromogen: DAB, Nuclei: Hematoxylin. Acetone fixed. Left: negative control, Right: Jurkat cell



Designer T cells. CEA-Fc (1:250), chromogen: DAB, Nuclei: Hematoxylin. Acetone fixed. Left: negative control, Right: Designer T cell

### d. tested different tissue storage method to resolve the tissue fall off issue for frozen tissues.

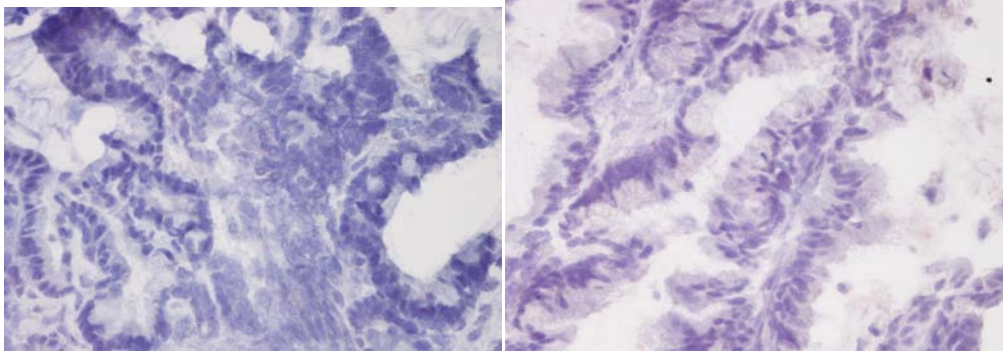
**Results: resolved the tissue falling off problem**

For the frozen sections, our tissue is very easy to fall out especially after H<sub>2</sub>O<sub>2</sub> blocking treatment. We found this was solved with increased drying time before the acetone fixation and after the acetone fixation. H<sub>2</sub>O<sub>2</sub> should also be made in methanol instead of distilled water or PBS.

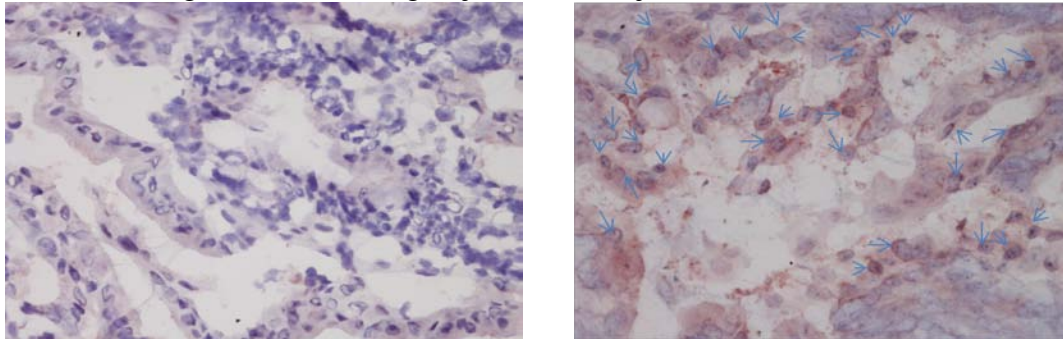
### e. tested juraket cell or designer T cell injected into tumor WI2 or CEA staining

**Results: find WI2 positive staining in designer T-cells injected into tumor, not in control slides. CEA-Fc has too much background staining on human tumor tissue. Cannot use CEA-Fc antibody on human tissues using 2 step methods**



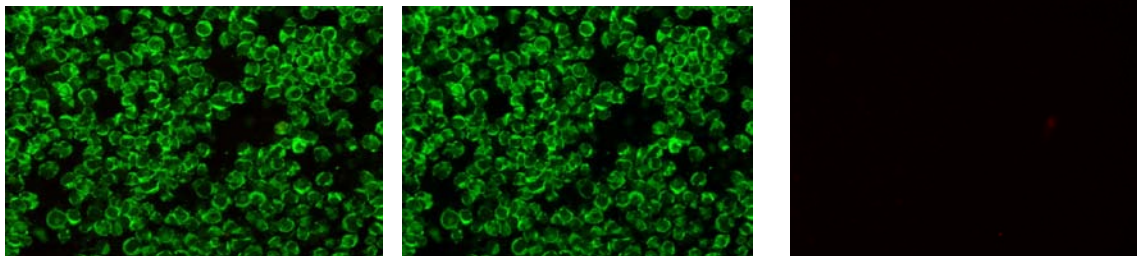


Jurkat cells in human Tumor. WI2 (1:25), chromogen: AEC, Nuclei: Hematoxylin. Acetone fixed. Left: negative control, Right: jurkat cells injected into tumor

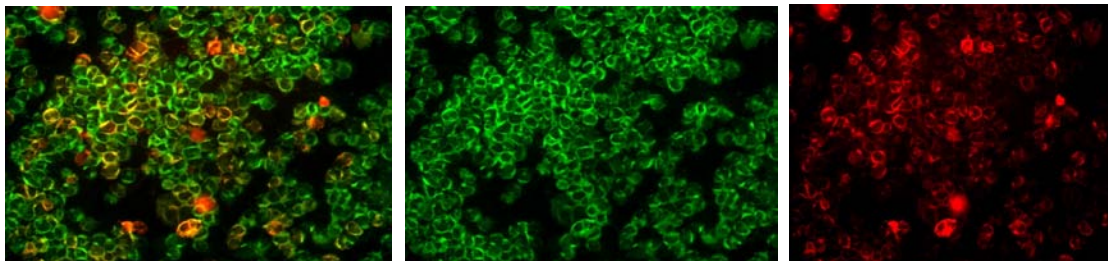


Designer T cells in human tumor tissue. WI2 (1:25), chromogen: AEC, Nuclei: Hematoxylin. Acetone fixed. Left: negative control, Right: Designer T-cell injected into tumor

### Immunofluorescence (IF) studies

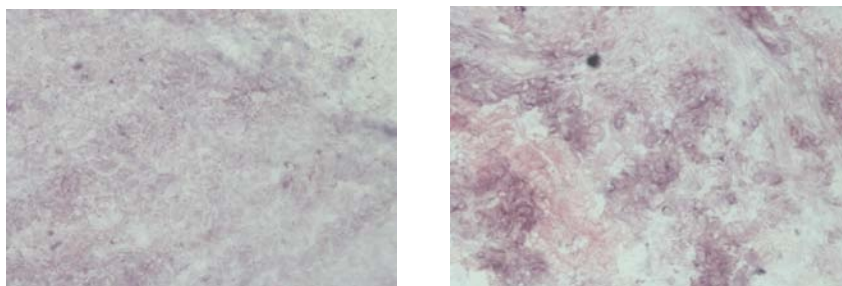


Jurkat cells. Acetone fixed. Left, CD3 (1:50, FITC),WI2 (1:50, Texas Red),middle: CD3 (1:50, FITC), Right: WI2 (1:50, Texas Red)

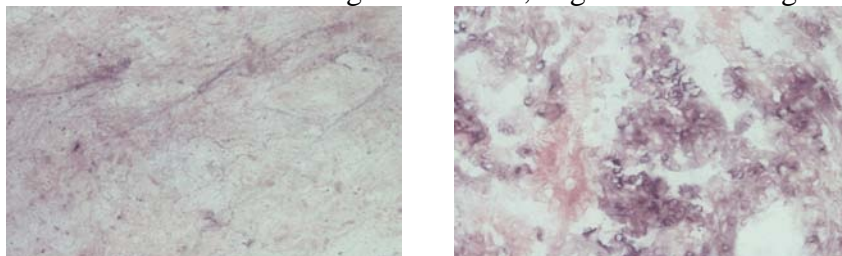


Designer T cells. Acetone fixed. Left, CD3 (1:50, FITC),WI2 (1:50, Texas Red),middle: CD3 (1:50, FITC), Right: WI2 (1:50, Texas Red)

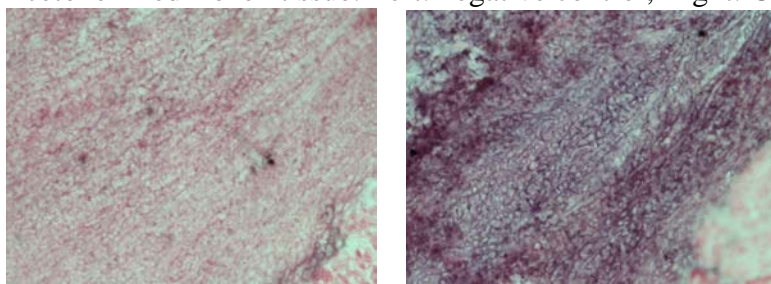
Right now, we are testing these 2 antibodies (WI2, CEA-Fc) to test mice tissues or human tumor tissues injecting jurkat cells and designer T-cells in frozen section or paraffin embedded section.



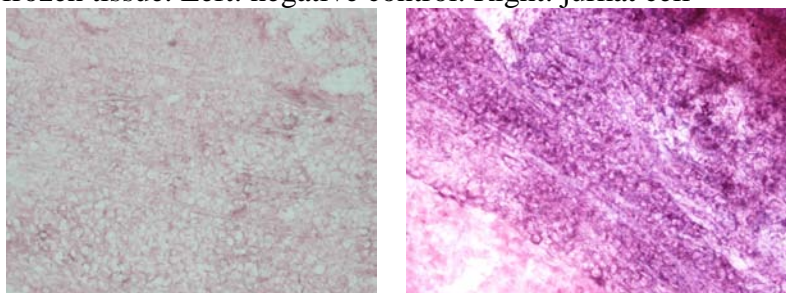
Jurkat cells injected into human tumor tissue. CD3 (1:50) staining. Chromagen: BCIP. Acetone fixed frozen tissue. Left:negative control, Right: CD3 staining



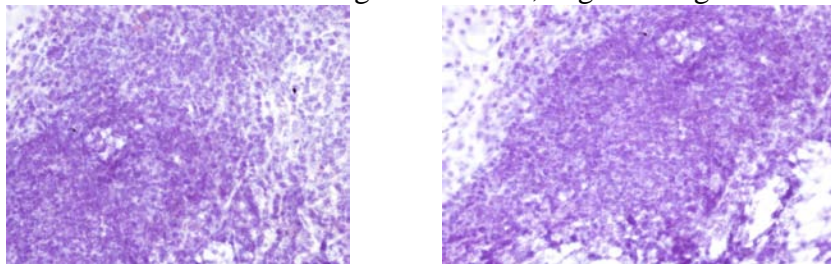
Designer T-cell injected into human tumor tissue. CD3 (1:50) staining. Chromagen: BCIP. Acetone fixed frozen tissue. Left: negative control, Right: CD3 staining



Jurkat cells injected into mouse kidney. CD3 (1:50) staining. Chromagen: BCIP. Acetone fixed frozen tissue. Left: negative control. Right: jurkat cell

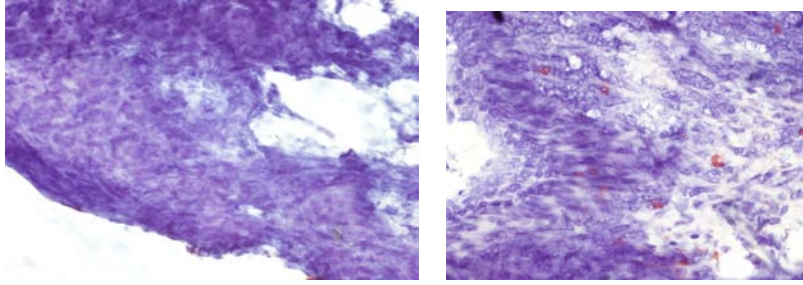


Designer T-cell injected into mouse kidney. CD3 (1:50) staining. Chromagen: BCIP. Acetone fixed frozen tissue. Left: negative control, Right: Designer T-cell



Jurkat cells injected into human tumor tissue. WI2 (1:50) staining, chromogen: AEC, Nuclei: Hematoxylin. Acetone fixed frozen tissue. Left:negative control, Right: jurkat cell

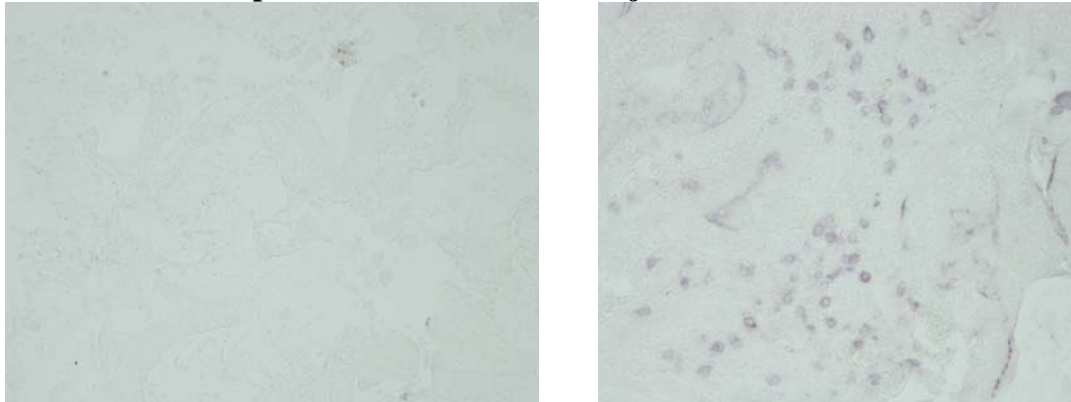




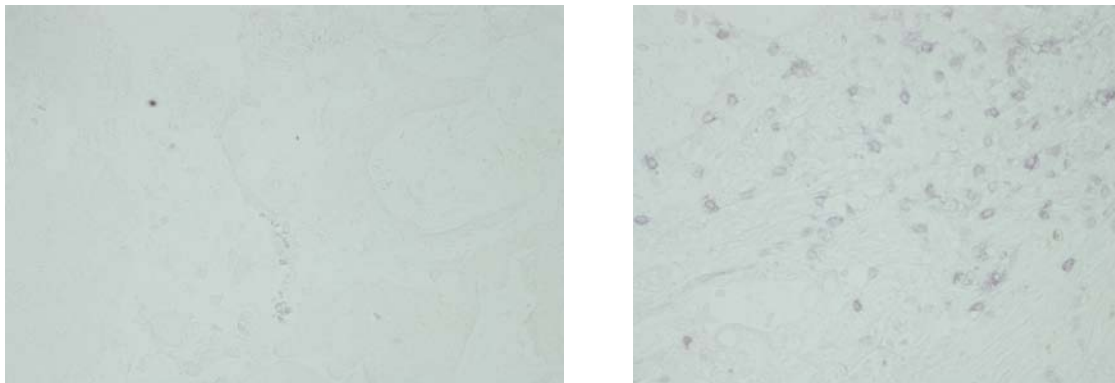
Designer T-cell injected into human tumor tissue. WI2 (1:50) staining, chromogen: AEC, Nuclei: Hematoxylin. Acetone fixed frozen tissue. Left: negative control, Right: designer T-cell

**f. tested juraket cell or designer T cell injected into tumor CD3 staining in paraffin embedded tissues**

**Results: find CD3 positive cells in both cells injected into tumor tissues.**



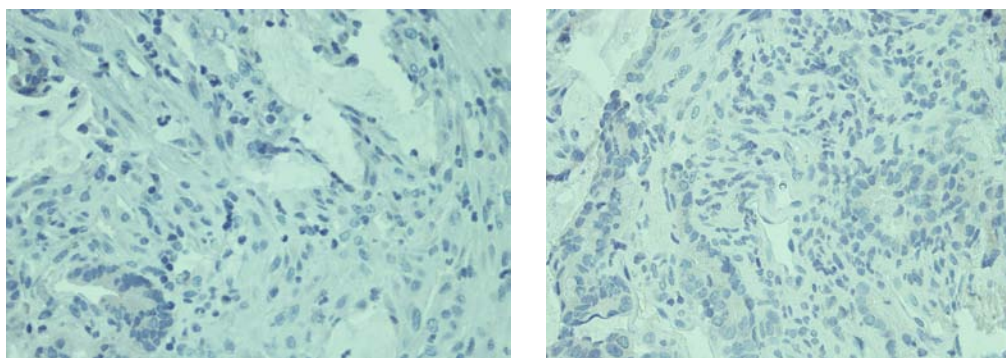
Jurkat cells in human Tumor. CD3 (1:50), chromogen: BCIP. **formalin fixed**. Left: negative control, Right: jurkat cells injected into tumor



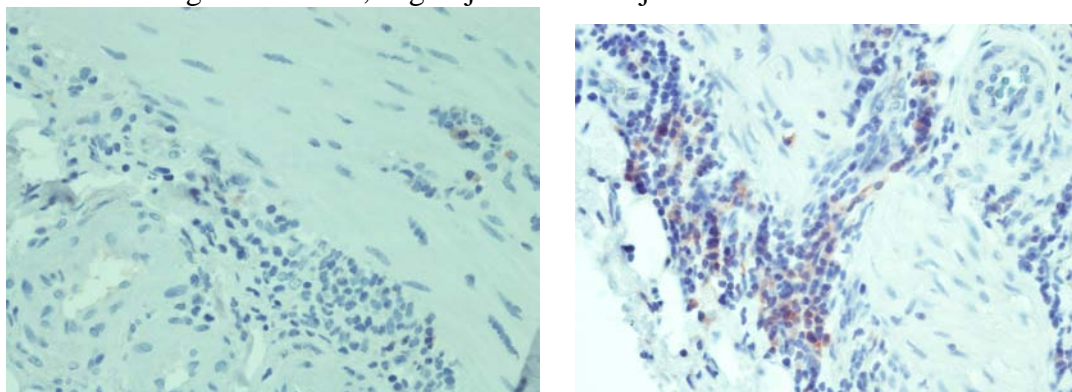
Designer cells in human Tumor. CD3 (1:50), chromogen: BCIP. **formalin fixed**. Left: negative control, Right: jurkat cells injected into tumor

**g. tested juraket cell or designer T cell injected into tumor WI2 staining in paraffin embedded tissues [new section]**

**Result: Find WI2 positive staining in designer T-cells injected into tumor, not in control slides.**



Jurkat cells in human Tumor. WI2 (1:25), chromogen: AEC, Nuclei: Hematoxylin. **formalin fixed**. Left: negative control, Right: jurkat cells injected into tumor



Designer T cells in human tumor tissue. WI2 (1:25), chromogen: AEC, Nuclei: Hematoxylin. **Acetone fixed**. Left: negative control, Right: Designer T-cell injected into tumor

## E. Administrative Note

During the first year, we were engaged with the HSRRB for harmonization of the protocol between the hospital and the Army. We also treated patients on the Phase Ia under separate funding that preceded the Phase Ib of this study. During this second year, the Phase Ia had its final patient treated, the Phase Ib was initiated, and optimization studies performed based on patient dose experience, continuing into the third year. These are now optimized. During this period, we have been careful to conserve resources for the clinical trial so that the treatment goals, though delayed, will not be hampered in their accomplishment.

## **Aim 2. Test ancillary procedures for improved persistence and activity of infused cells**

### A. Testing engraftment protocols

#### 1). Rationale.

After the current Phase Ib and Phase II studies, we will explore dTc engraftment, by infusing dTc after high dose chemotherapy. This leads to a massive expansion of the cells in vivo, with marked increases in activity/potency. However, it can also lead to serious toxicities and patient deaths if the target antigen has not been validated as “safe”. The Phase Ib tests as we are applying them, will assess this safety. In anticipation of this effort, we have undertaken preclinical work to test some of the potential of this method.

#### 2). Anti-tumor animal model of anti-CEA Tandem dTc with engraftment

a) *Verifying anti-CEA IgTCR and anti-CEA Tandem CARs are functional in mouse T cells.*  
Results show that mouse T cells transduced with anti-CEA IgTCR and Tandem will activate to produce IL2 and exhibit specific cytotoxicity to kill MC38-CEA tumor cells but not MC38 cells.

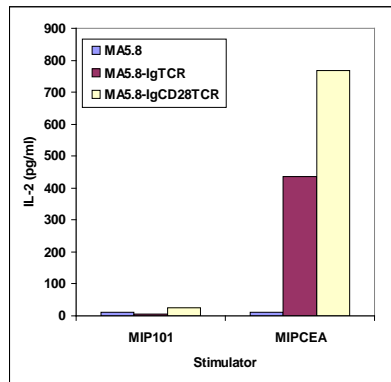


Figure 8. IL2 production by 1<sup>st</sup> and 2<sup>nd</sup> gen anti-CEA human CARs in mouse T cell line (MA58). IgTCR = 1<sup>st</sup> gen; IgCD28TCR (Tandem) = 2<sup>nd</sup> gen.

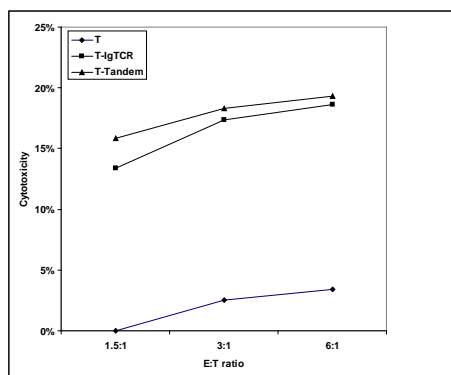
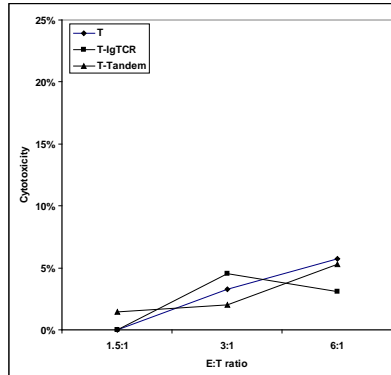


Figure 9. Cytotoxicity by 1<sup>st</sup> and 2<sup>nd</sup> gen anti-CEA human CARs in normal activated mouse T cells. T = unmodified mouse T cells; IgTCR = 1<sup>st</sup> gen; Tandem (IgCD28TCR) = 2<sup>nd</sup> gen. Left panel, MC38 targets (antigen -ve); Right panel MC38-CEA (antigen +ve)

b) *Performing anti-tumor animal model with engraftment.*

The in vivo antitumor activities of the anti-CEA IgTCR and Tandem dTc were examined in a mouse engraftment model.  $50 \times 10^6$  mouse T cells transduced with anti-CEA IgTCR and Tandem CARs were injected i.v. into 350 rads  $\gamma$ -irradiated mice that inoculated with  $1 \times 10^6$  MC38 and MC38-CEA tumor. Our preliminary results show that compared to negative control mice that were treated with unmodified T cells, MC38-CEA tumor growth was totally suppressed in anti-CEA Tandem dTc treated mice and partially suppressed in anti-CEA IgTCR dTc treated mice. To be repeated with more animals for statistical validity.

## B. Utility of IL15

### 1). Ex vivo expansions with IL15

We previously showed benefit of IL15 + IL2 for a super-proliferation of activated T cells, comparable to the expansions we obtained with high dose IL2 (Fig.10).

Additional tests have been performed with the IL2 and IL15 supplementation of cultures of activated T cells (aTc). T cells were isolated and grew in different conditions, high concentration of IL2 (3000 IU/ml),

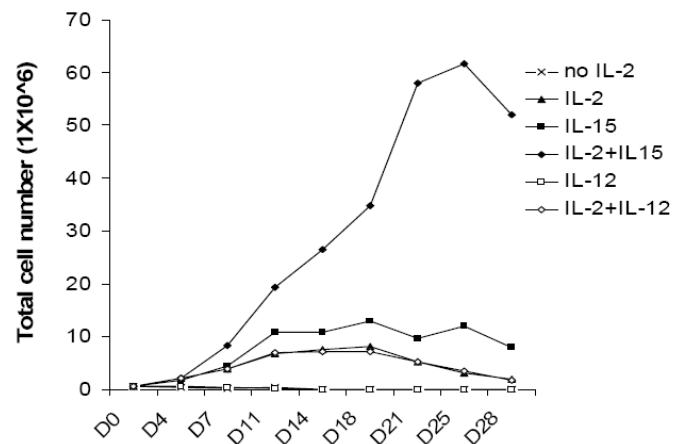


Figure 10. Activated T cell expansion in cytokines. See text. (A Lo & RPJ, unpubl. results)

normal concentration of IL2 (300 IU/ml) and IL15 (10 ng/ml). For this donor, there is no synergy for the growth curve between normal concentration of IL2 and IL15 that we previously reported.

This may have to do with the activation state of the cells when placed in supplemented media, or even the donor source of cells. We will test these features. Also of interest for cytokine response, are data showing better expansion with high dose IL2 (3000 IU/ml) versus standard IL2 (100-300 IU/ml). Because IL15 and IL2 share the beta and gamma chains of their respective receptors, we wish to examine if the receptor occupancy of the shared receptor chains accounts for the benefit of IL2+IL15, in which case high dose IL2 as a single agent may accomplish the same thing. This will require careful quantitation of the receptors and their occupancy status.

To examine the cytokine receptor expression for judging the effects of these cytokines, IL2Ra, IL2Rb and IL15Ra were checked by FACS analysis. IL2R expression of T cells in all conditions was low after two weeks. It is necessary to examine at earlier time points for the IL2R expression profile.

## 2). In vivo expansions: IL15 as a substitute for lymphodepletion conditioning protocols to create engraftment

Under circumstances of lymphodepletion, we showed that IL15 levels rise sharply during the period of lymphopenia until restoration of counts, as seen in Figure 11. It is plausible that we can bypass this costly and hazardous maneuver of high dose chemotherapy conditioning for induction of lymphopenia to foster dTc engraftment and simply administer IL15 concurrent with the dTc infusion to mimic what happens after the conditioning step.

The mechanisms of T cell engraftment with dTc infusion after lymphodepletion conditioning is speculated to be due to high levels of IL15 and IL7 in the lymphopenic state after conditioning. The conditioning protocols are dangerous with real death rates from neutropenia and infections. We wish to explore the option of using IL15 and/or IL7 to achieve the same thing without the hazards.

In a parallel study with conditioning (in prostate cancer), we assessed the IL7 and IL15 levels after conditioning in five patients. IL7 was not reliably detected, but IL15 was steady for the duration of lymphopenia and then declined to zero after lymph recovery (Figure).

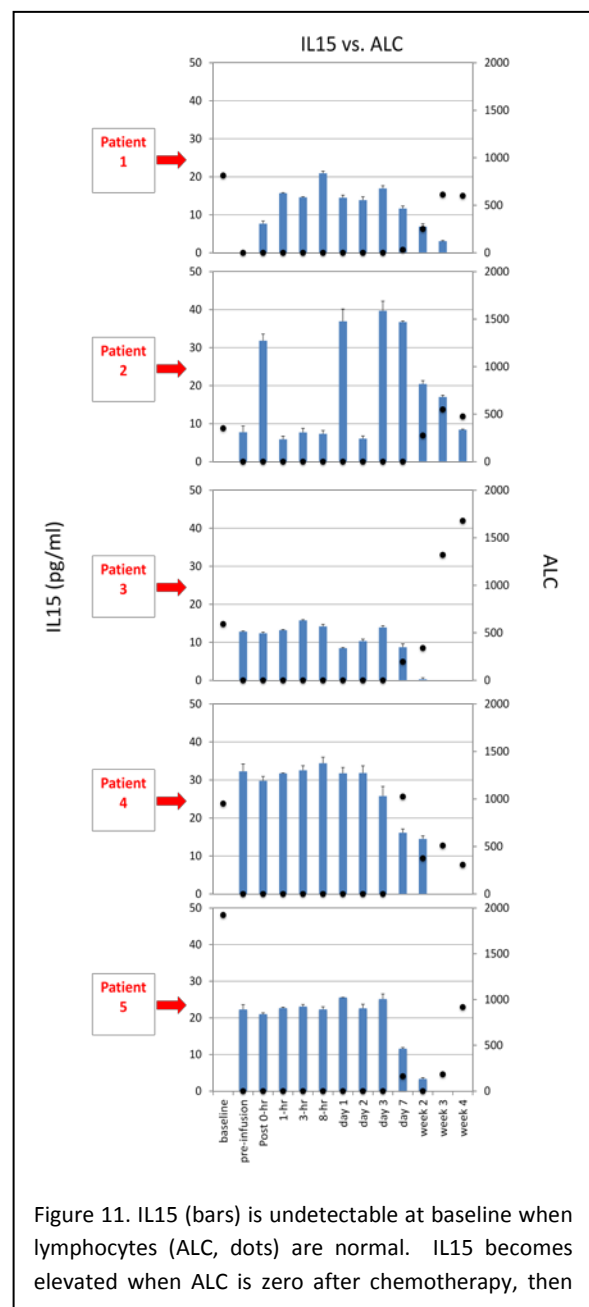


Figure 11. IL15 (bars) is undetectable at baseline when lymphocytes (ALC, dots) are normal. IL15 becomes elevated when ALC is zero after chemotherapy, then

The key aspect of IL15 that is of interest to us is that it promotes T cell expansion in the absence of activation (termed “homeostatic expansion”), allowing high levels of cells to be generated, without IL2. We will plan experiments to define this role in mice and attempt to reproduce the levels in mice that occur during lymphopenia to determine if the same levels occur and if providing the same levels to normal animals causes a similar expansion of infused cells. If successful, this approach could save expense (\$50,000 per patient) and improve safety over present conditioning regimens.

We will devise animal tests of this proposal to pursue in the coming year.

### **Aim 3. Devise and preclinically test next (3<sup>rd</sup>) generation product**

#### ***Aim 3.1. Create and test additional co-stimulatory configurations for CAR***

Costimulation plays an essential role in the survival and expansion of T cells after antigen encounter. Our second generation product of the Phase Ib study is complemented with CD28 costimulation.

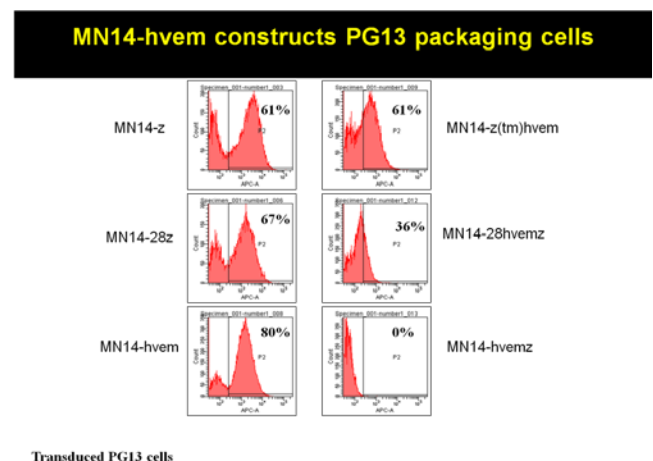
We proposed a plan to perform high through-put testing of second co-stimulatory molecules in the format of Ig-CD28-X-zeta, in which Ig represents the MN14 antibody recognition domain, and X is the second co-stimulatory molecule. (CD28 is the first.) Constructs have been prepared for all of the following co-stimulatory molecules. These include X = HVEM, 4-1BB, ICOS, OX40, and CD27. This work was performed by Dr Bais.

MN14-X  
MN14-z(tm)-X  
MN14-28-X-z  
MN14-X-z

We previously reported early data on the HVEM constructs. Controls are MN14-zeta (1<sup>st</sup> generation) and MN14-CD28-zeta (2<sup>nd</sup> generation). These both express well in PG13 cells, our vector producer cell line. With HVEM, we characterized the following.

MN14-HVEM  
MN14-z(tm)-HVEM  
MN14-28-HVEM-z  
MN14-HVEM-z

Of these, the MN14-HVEM expressed the best, followed by MN14-z(tm)-HVEM (using the zeta TM domain), then MN14-28-HVEM-z, and non-expressing MN14-HVEM-z. The desired configuration with 3 signals in the single construct was thus shown to be problematic for expression. The origin of this problem is not obvious, but it suggests that this construct cannot be effectively tested in our T cells.





As an alternative, we are co-expressing MN14-28-z and MN14-HVEM in the same vector, separated by a 2A sequence for two-gene expression. This applies a novel procedure devised in this laboratory to suppress homologous recombination between the repeated MN14 sequences that leave one gene rather than two. Dr Bais undertook a mutagenesis procedure that wobbles the bases for every amino acid possible in one of the repeated elements.

We have now prepared a large number of constructs. These are shown in the following table, with more to follow. In particular, we are interested in further attempts with the CD2 construct which had problems with expression in the Aim 3.2 studies reported previously.

1.	IgTCR-z
2.	IgTCR-28z
3.	IgTCR-hvem
4.	IgTCR-hvemz
5.	IgTCR-28hvemz
6.	IgTCR-z(tm)hvem
7.	IgTCR-41BB
8.	IgTCR-OX40
9.	IgTCR-ICOS
10.	IgTCR-CD27

We previously reported functional tests were stalled by mycoplasma contamination of one of the parental cell lines for making VPCs. New VPCs were prepared, but there were still difficulties with transduction. We then obtained again new parental helper lines to re-examine this matter, and VPCs re-prepared. The titers were improved.

During the past year, we had recruited Dr Agnes Lo, a researcher who formerly trained with the PI. Dr Lo is outstanding with the biologic tests of these agents, which are not trivial to accomplish. Dr Bais was to complete the molecular constructs for Dr Lo to complete testing, but Dr Bais left the lab during this past year. We have recruited a new research, Dr Xiuyang Guo. He will join the group in August 2012 to resume the molecular work of this project, and collaborate with Dr Lo in their completion. Dr Guo's resume is attached in the appendix.

### **Aim 3.2. Create and test products to avoid need for IL2 supplementation**

There are three parts to this Aim:

- 3.2.1) to examine Signal 3 (LFA1 and CD2) for their capability to yield sustained IL2 on re-stimulation with antigen,
- 3.2.2) to express IL2 constitutively from a promoter within the transferred transgene, and
- 3.2.3) to express anti-apoptotic genes the make cells resistant to IL2 withdrawal.
- 3.2.4) [new subaim] to understand mechanism for IL2 shutdown on repeated stimulation

We have made progress on subaim #3.2.1, and are finalizing a manuscript for publication. (We were waiting for the contributions of a former scientist, Dr Gomes, to complete the manuscript. She has promised to focus on this promptly.) We previously had shown that IL2 secretion with Signal 1+2 was abundant, but that IL2 release was lost on successive antigen restimulations [1].



We recently published that IL2 was essential for in vivo responses with 2<sup>nd</sup> generation designer T cells in animal models, despite the high IL2 secretion on antigen contact [2]. Our human trial with dTc in prostate cancer suggests the same [3]. Our hope had been that Signal 3 would overcome the block to IL2 secretion on restimulations to help the T cells replicate in vivo without exogenous IL2 stimulation. [NB: the co-stimulatory molecules of Aim 3 are all considered Signal 2, although in tandem with zeta and CD28 will provide three signals. We reference CD2 and LFA1 as Signal 3 being qualitatively different, based on data of Sprent and Schlom. See grant proposal for references.]

In our experiments, we stimulated resting and activated T cells with Signal 1 or Signal 1+2 and assessed the impact of adding Signal 3 on IL2 and gamma interferon secretion. Whether with antibody stimulation or 3<sup>rd</sup> generation CAR signaling, we found improved IL2 production with 3 signals, but it was found still not to be self-sustaining. Thus, the hypothesis of sufficiency for IL2 production with three signals was falsified. This shifts the hope for avoiding systemic IL2 costs and toxicities to the subsequent subaims.

We had good expression and function of the LFA1 constructs, but, as stated above, we had problems to express a CD2 based construct. We will look at this in the context of the costimulation experiments above. Our tests showed that the IL2 secretion signal was primarily from the LFA1 beta chain, and not the alpha chain. These data were previously unknown, and speak to the outside-in signaling of the LFA1 that has been primarily thought of as an adhesion molecule.

We have done nothing further with the subaims 3.2.2 and 3.2.3 that address constitutive secretion of IL2 and anti-apoptosis genes. These will follow. Crucially, however, we have added a further subaim to get at the question of IL2 secretion and why it shut off.

#### Aim 3.2.4. The signaling features of IL2 secretion shutdown.

The failure of 3 signals that were thought the best chance for IL2 sustained secretion now begs the question to understand the mechanisms of this shutdown. IL2 is first abundant, then rapidly decline with restimulation of the T cells. This suggests an active repression mechanism, either via phosphorylation or dephosphorylation of intermediates in the signaling cascade, or chromatin remodeling or transcription factor alterations. Although the pathways to activating IL2 production are well studied, the shutdown of IL2 production has not been examined. Once this is understood, there may be ways to regulate it so that IL2 continues for as long as the T cells encounter antigen. Both signal transduction and transcription analysis will be performed.

We previously reported the recruitment of a new junior faculty member, Patrycja Dubielecka, PhD, as Director, Signal Transduction Lab. In the prior annual report, her recruitment was indicated. She has been the prime collaborator in the IL2 studies that follow. This is adapted from her reports.

**Rationale:** CEA specific tandem designer T cells (dTc), upon exposure to antigen, undergo population expansion, which is accompanied by the enhancement in cytokines secretion. Repeated exposure to antigen, however, can lead to contraction of dTc numbers and a shutdown of IL2 secretion. The relationship between these two phenomena and their mutual dependence and sequential occurrence remain to be established.

The aims of this project are a) to identify the sequence of events leading to decrease in the dTc number and b) to define the pathway(s) governing the IL2 production shut down in CEA specific tandem dTc.

In our approach to understand the mechanisms of IL2 shutdown, we use three experimental systems (i) Resting cells, (ii) Primed CD4 and CD8 cells, (iii) Modified dTc. We believe that the data obtained from the analyses conducted on resting and primed cells will help to understand the mechanisms of IL2 shutdown regulation in dTc in the ultimate goal to block them. Below, we present results obtained from experiments on resting cells.

**[NB: in the following section, the transfer of information was with Figure numbers attached to them relevant to each presentation of the data, and because of the nature of the picture files, these embedded numbers cannot be changed by me. The numbering and figures are local to the text adjacent. The information should be comprehensible, regardless.]**

(a) Resting T Cell experimental system.

**Experimental outline:** Resting CD4/CD8 cells were isolated from peripheral blood, stimulated for one week, every other day (3x) with the CD3/CD28 engaging antibodies, to generate signal 2 response (relevant to second generation dTc) (Fig1.). Analyses of IL2 secretion, proliferation, apoptosis, STAT pathways stimulation and change in transcription factors profile were conducted, data from analyses done on two separate donors are presented.

Fig.1.

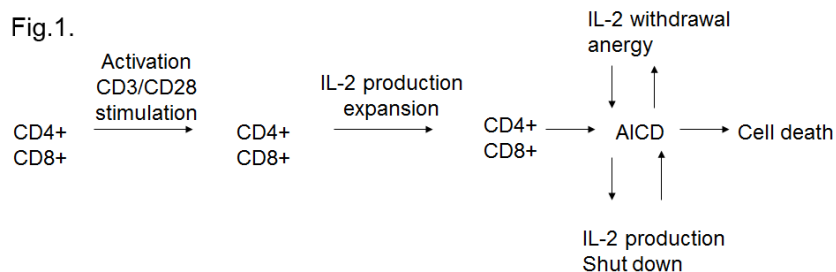


Fig.1. IL2 shutdown – experimental approach: Resting CD4/CD8 cells, isolated from peripheral blood, will be subjected to three step-activation by anti CD3/anti-CD28 Ab (2<sup>nd</sup> signal). IL2 production, proliferation, apoptosis, STAT3/5 pathways and changes in transcriptional profile of

**IL2 production:** We have observed that IL2 secretion by resting cells is essentially completed after the first stimulation on day 0, with no additional IL2 in the culture after subsequent (day 2 and day 4) re-stimulations (Fig.2), consistent with the dTc data (Emtage et al, 2008).

**Fig. 2.** IL-2 production in CD3/CD28 activated resting cells

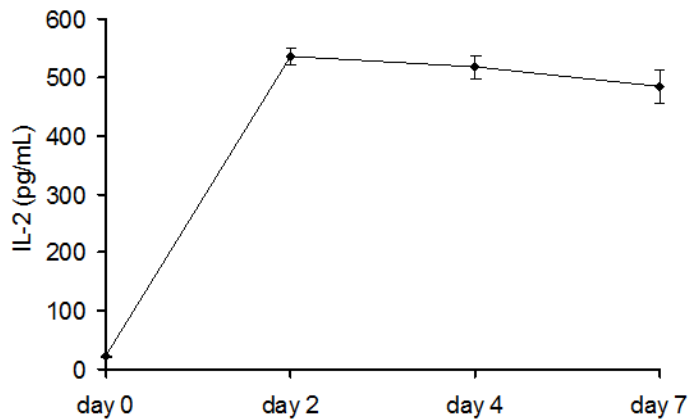


Fig.2. IL2 secretion is complete after first stimulation of resting cells by anti-CD3/anti-CD28 Ab. Cumulative IL2 is represented with IL2 in culture at each time-point prior to the next stimulation. The slight decline could be due to IL2 consumption by the activated T cells.

**IL2 receptor expression:** For IL2-dependent responses, both the production of IL2 and expression of the IL2R must contemporaneously occur within the same microenvironments. Therefore, we analyzed the expression of IL2R, following the inducible IL2Ra that contributes to form the high affinity IL2R together with the constitutively expressed beta and gamma-c chains. We noted that peak IL2Ra expression is on day 4 (Fig.3), after the peak of IL2 secretion that is complete before day 2. The role of IL2 versus activation to mediate the IL2Ra expression needs to be investigated.

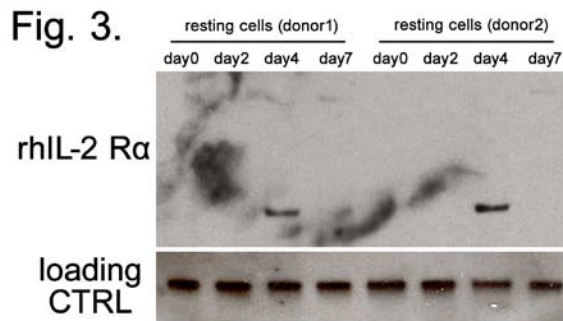
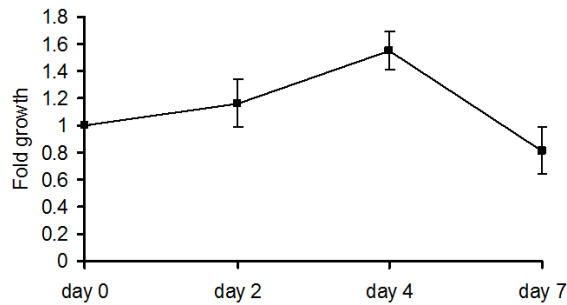


Fig.3. IL2Ra has peak expression on day 4 after initial and then re-stimulation by anti-CD3/CD28 Ab, then declines. Western blot of cells isolated at times indicated. Data presented for re-stimulated resting cells isolated from 2 donors (two biological replicates).

**Proliferation:** We analyzed the proliferative potential of stimulated and re-stimulated CD4/CD8 resting cells. We observed that enhancement of IL2 secretion is leading not only to the expression of IL2 but it correlated with enhancement of growth and proliferation of re-stimulated CD4/CD8 resting cells. Stimulated population reached peak at day 4 and declined (Fig. 4A). Stimulated cells expanded on day 2 and 4 and contract in number by day 7 of antigen exposure. Nevertheless, cells are continuing to replicate, as seen by the emerging daughter population 2 in Fig.4B, showing waves of T cell division. The difference is made up by apoptosis in another portion of the cell population (discussed below).

**Fig. 4.A** Growth of CD3/CD28 activated resting cells



**Fig. 4B** Proliferation of CD3/CD28 activated resting cells

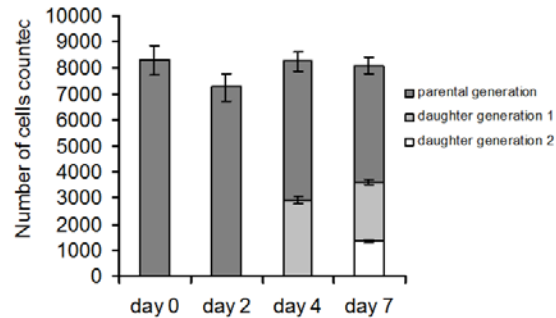
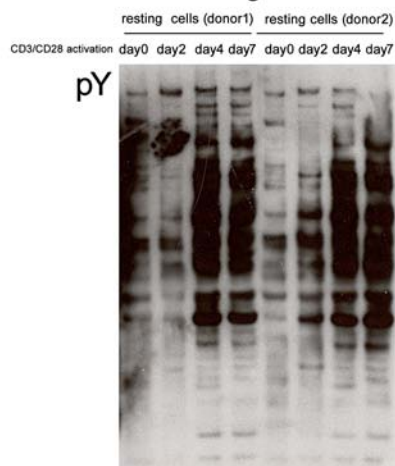


Fig.4AB. The proliferative potential of stimulated resting cells reaches its maximum on day 4 upon stimulation as evaluated by (A) simple viable cell count. (B) CFSE tracking of daughter populations shows that a population continues to expand (e.g. daughter generation 2) while the total cell number has declined on day 7.

**Signaling pathways:** The strengthening of a general phosphor-tyrosine phosphorylation at day 4 after re-stimulation of resting CD4/CD8 cells (Fig.5A), strongly suggested activation of IL2 dependent signaling cascade via Src family of tyrosine kinases to STAT/ERK. Indeed we observed enhancement of the phosphor ERK and STAT. Interestingly, the increase in cellular phosphor-signal corresponded with enhancement in proliferative potential at day 4 upon stimulation. Compare Fig. 4A and 5.

**Fig. 5A** Phospho-tyrosine signal in CD3/CD28 activated resting cells



**Fig. 5B**

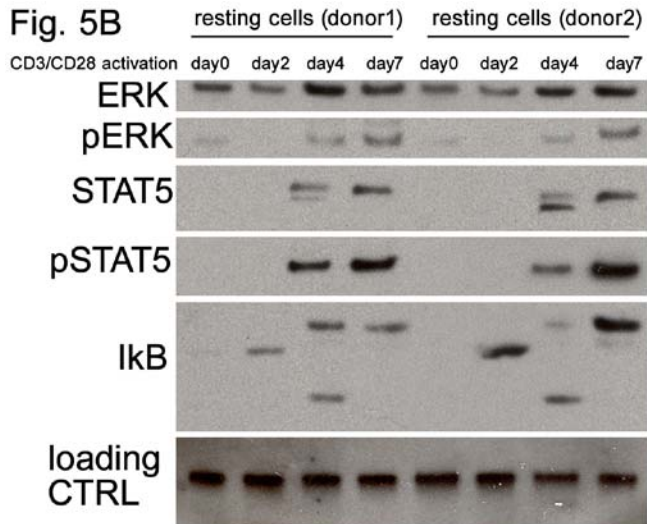


Fig. 5. Enhancement of (A) general and (B) specific phosphorylation signals on tyrosines (pY) is high on day 4 and day 7, compatible with IL2 dependent activation of scr kinases leading to activation of Erk and Stat5 pathways. Observed enhancement in phospho-Erk and phospho-Stat5 corresponded with enhancement of proliferative potential of re-stimulated resting cells.

**Apoptosis:** Initial enhancement in IL2 production and proliferation is followed by apoptosis in a portion of the CD3/CD28 stimulated resting cells. Analysis of the percentage of apoptotic cells confirmed increase in number of apoptotic cells on day 7 post antigen exposure, to 45% (Fig.

6A). Immunoblotting of expression of the panel of pro-apoptosis regulatory proteins: Bcl-xl/s, Bid, Granzyme B confirmed increase of expression of most of these proteins on day 7<sup>th</sup> of antigen induced activation (Fig. 6B). Decrease in the levels of anti-apoptotic proteins Bcl-2, XIAP and FLIP was observed at day 7 (Fig. 6C).

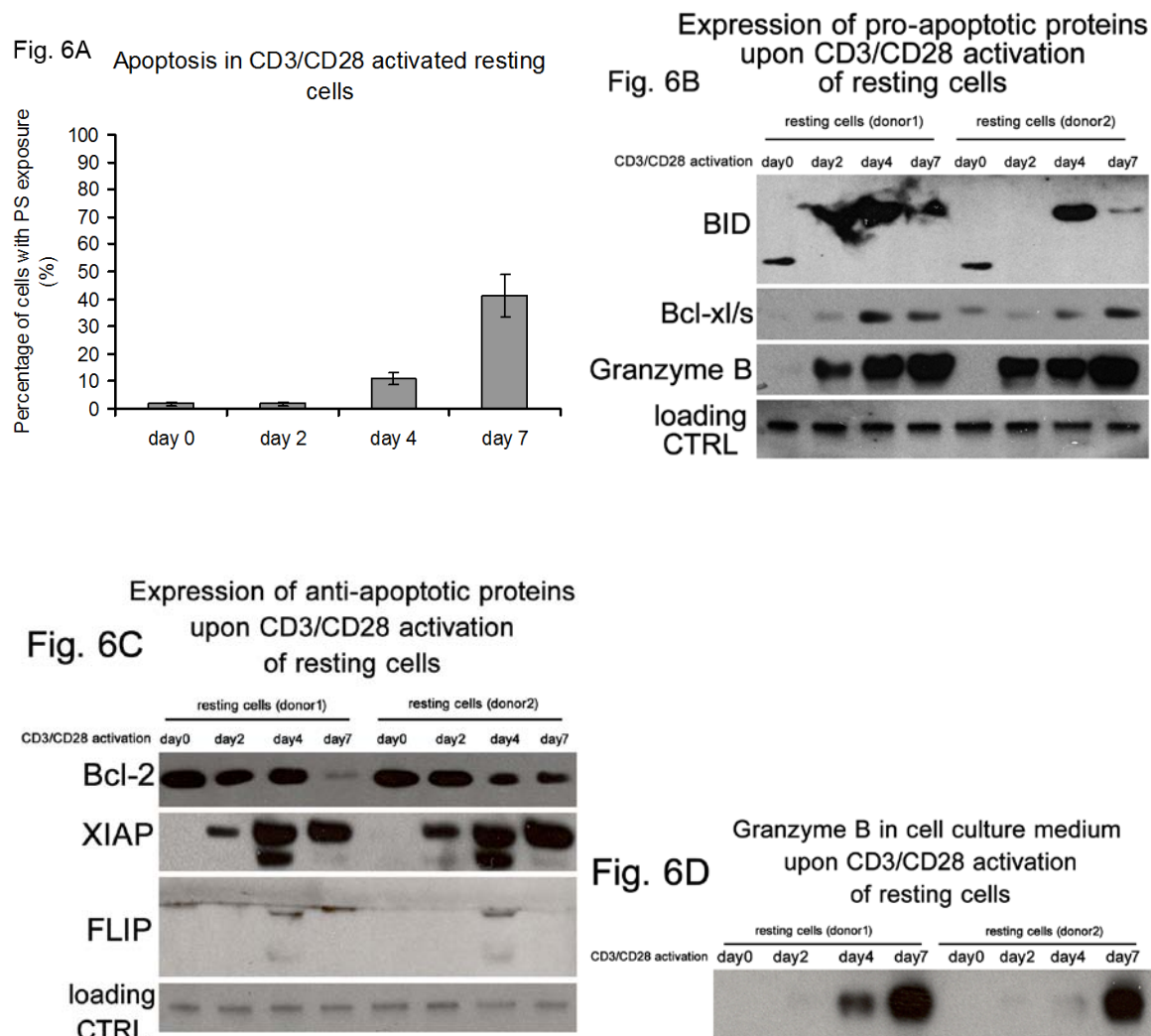


Fig. 6. Apoptosis after stimulation. (A) Evaluation of the number of cells with the apoptotic exposure of PS, as detected by Annexin V, showed increase in the apoptotic cells on day 7 upon re-stimulation of resting CD4/CD8 cells. Increase of the amount of pro-apoptotic (B) BID, Bcl-xl/s and Granzyme B and decrease of anti-apoptotic (C) Bcl-2, XIAP and FLIP were noted on day 7 post-stimulation, compatible with contraction of previously expanded stimulated cells. (D) The amount of secreted Granzyme B was increasing in the cell culture medium, suggesting either enhancement of secretion of this protease, or lysis of apoptotic cells.

**Translational impact:** Ultimate goal of this project is to introduce the specific modification to the CEA specific dTc, which would counteract IL2 production shut down and population contraction of the dTc upon prolonged exposure to the antigen. Production of such cells will significantly prolong their systemic persistence and reactivity.

## (b) Primed T cell experimental system

**Experimental outline:** PBMCs were isolated from blood of healthy donors (n=4), PBMCs were monocyte depleted by adherence in overnight overnight culture, amounts of lymphocytesCD4/CD8 were assessed by immunostaining and the ration of both populations was 1:1. Cells were cultured for 5 days in expansion RPMI-based medium containing 300 U/mL of IL2 and 30 ng/mL of anti-CD3 antibody (OKT3), priming. These primed cells were then subjected to one week stimulation, every other day (3x) with the CD3/CD28 engaging antibodies, to generate signal 2 response (Fig1.). Analyses of IL2 secretion, IL2Ra expression, proliferation, apoptosis, TCR/IL2 induced pathways status and change in transcription factors profile were conducted, data from analyses conducted on four biological replicates (four donors) are presented.

Fig.1.

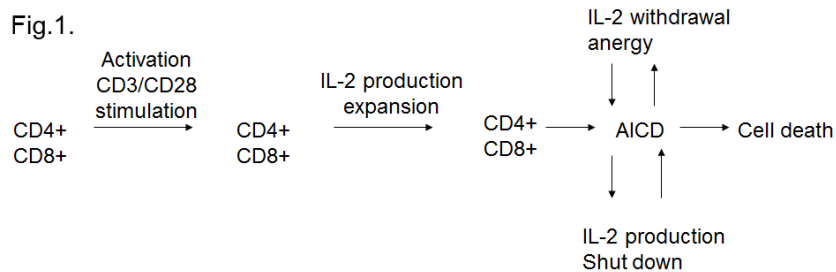


Fig.1. IL2 shutdown - experimental approach: Primed CD4/CD8 cells, isolated from peripheral blood, are subjected to three step-activation by anti CD3/anti-CD28 antibodies (2<sup>nd</sup> signal). IL2 secretion, IL2Ra expression, proliferation, apoptosis, TCR/IL2 induced pathways status and change in transcription factors

**IL2 production:** We have observed the maximum secretion of IL2 by stimulated resting cells on day 2<sup>nd</sup> after first stimulation, with progressing decrease of IL2 secretion noted on days 4<sup>th</sup> and 7<sup>th</sup> (Fig.2).

Fig. 2.

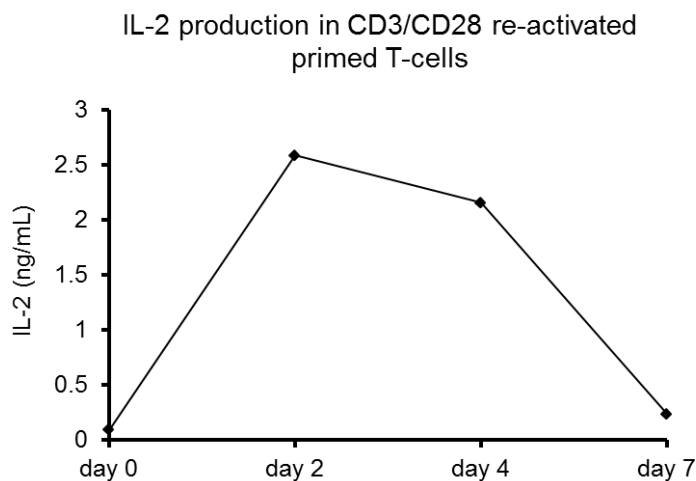


Fig. 2. Maximum IL2 secretion is observed on 2<sup>nd</sup> day after initial stimulation of primed cells by anti-CD3/anti-CD28 antibodies. Despite the additional 2 stimulations on day 2<sup>nd</sup> and 4<sup>th</sup>, continuous decrease in IL2 detection is observed. Data from four biological replicates.

**IL2 receptor expression:** For IL2-dependent responses, both the production of IL2 and expression of the IL2Ra must temporally occur within the same microenvironments. Therefore we have analyzed the expression of IL2R. We have noted that priming in the presence of IL2 led to continuous expression of IL2Ra throughout the re-stimulation experiments, with the highest expression of IL2 on day 0 of anti-CD3/CD28 re-stimulation and the lowest on day 7<sup>th</sup> (Fig.3).



Fig. 3.

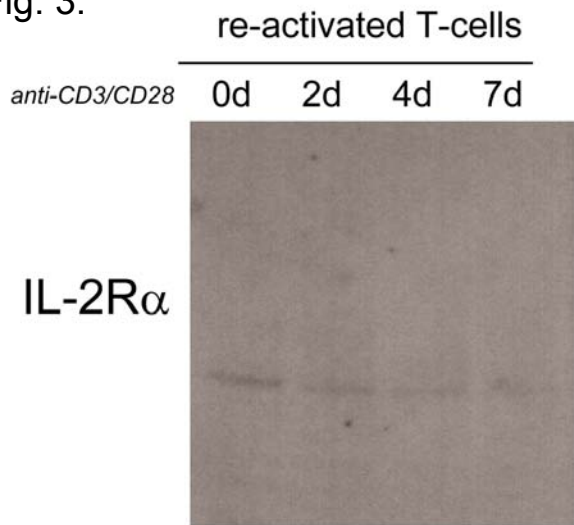


Fig.3. Analyses of expression of IL2Ra revealed that IL2 receptor is expressed continuously throughout the re-stimulation treatment with its highest amount detected on day 0 and lowest on day 7<sup>th</sup>. Date represent four biological replicates.

**Proliferation:** We have analyzed the proliferative potential of re-stimulated CD4/CD8 primed cells. We have observed that peak of IL2 secretion is accompanied with the highest number of cells observed on day 2<sup>nd</sup> of re-stimulation. Re-stimulated population reached peak at day 2<sup>nd</sup> and declined (Fig. 4AB). Re-stimulated cells expanded on day 2<sup>nd</sup> and 4<sup>th</sup> and contract in number on day 7<sup>th</sup> of antigen exposure, as it is similarly observed for antigen exposed designer T cells. Notably we have also observed increased proliferation rate for primed cells in comparison to resting cells presented before.

Fig. 4.

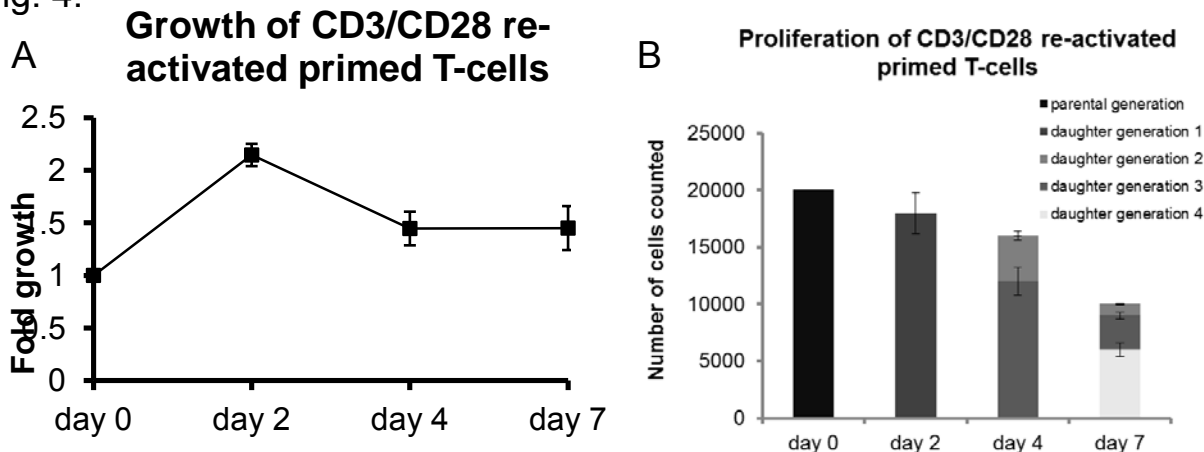
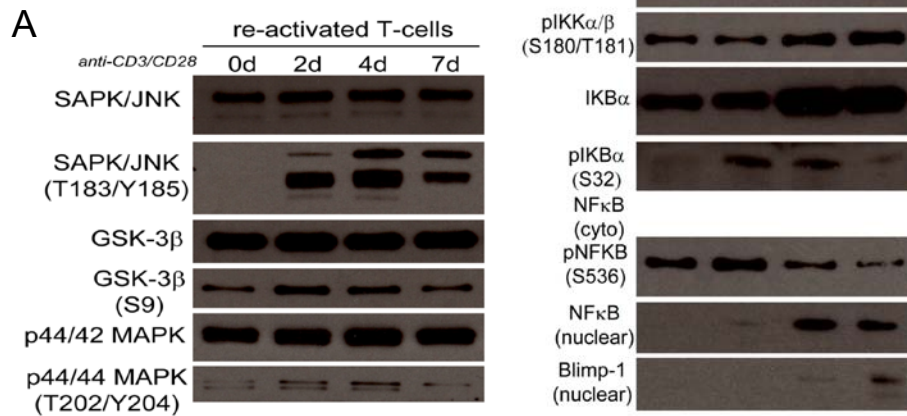


Fig.4AB. The proliferative potential of re-stimulated primed cells reaches its maximum at day 2<sup>nd</sup> upon re-stimulation as evaluated by both simple cell count (A) and CFSE tracking of daughter populations (B).

**Signaling pathways:** As the cells are primed in the presence of IL2 and re-stimulated by the engagement of TCR and co-stimulatory CD28, anticipated activation of both TCR and IL2 signaling pathways in the re-stimulation experiments. We have analyzed three major pathways, MAPK, Jnk/SAPK and PI3, activation of which is common for both TCR and IL2R engagement. All three pathways were activated on day 2<sup>nd</sup> and 4<sup>th</sup> of re-stimulation as evaluated by the phosphorylation signal on SAPK, GSK3b and Erk1/2 (Fig.5).

**Fig. 5**

Fig. 5 AB. Engagement of SAPK/JNK, MAPK and PI3k pathways is observed in re-stimulated cells on day 2<sup>nd</sup> and 4<sup>th</sup> of the experiment, suggesting engagement of TCR and IL2R pathways. Activation of STAT5/NFkB pathway is observed on day 2<sup>nd</sup> and 4<sup>th</sup> and shuts down on day 7<sup>th</sup>, possibly due to the up-regulation of Blimp-1.



We have also evaluated the state of activation of major responders to IL2 secretion: STAT5 and STAT3. We observed the highest stimulation of STAT5 on day 2 of re-stimulation; which corresponds with the peak of IL2 secretion. STAT5 phosphorylation diminishes on day 4 and 7 upon re-stimulation. We also analyzed the status of release of NF-κB from its inhibitory complex with IκB. We have detected the activation of IKK kinase leading to phosphorylation of IκB, which in turn

dissociates from NFκB and allows for its nuclear translocation. Indeed, in the course of experiment we have observed diminishing fraction of cytoplasmic NFκB and increasing fractions of nuclear NFκB. Expression of NFκB should lead to up-regulation of IL2 expression, however as we observed no IL2 secretion increase at day 7<sup>th</sup> of re-stimulation, we conclude that Blimp-1, which is overexpressed on day 7<sup>th</sup> of re-stimulation, counteracts the IL2 expression. Blimp-1 is expressed on chronically activated short term effector cells and CD4/CD8 cells in this experiment have full characteristics of such cells, what is additionally supported by the high expression of IL2Ra.

**Apoptosis:** Initial enhancement in IL2 production and proliferation is followed by apoptosis in CD3/CD28 re-stimulated primed cells. Analysis of the percentage of apoptotic cells confirmed increase in number of apoptotic cells on day 7<sup>th</sup> of antigen exposure (Fig. 6). Interestingly we do observe bi-phasic increase in number of apoptotic cells, with first increment on day 2<sup>nd</sup> of re-stimulation and the second on 7<sup>th</sup>. This suggests that some sub-population of CD4/CD8 cells is contracting perhaps as a result of IL2 withdrawal or aggressive re-stimulation conditions. Immunoblotting analyses of expression of the panel of pro-apoptosis regulatory proteins: Bim, Bid, Granzyme B confirmed increase of expression of most of these proteins on day 7<sup>th</sup> of antigen induced activation (Fig. 6B). Some increase of their production is also observed on day 2<sup>nd</sup> of re-stimulation. Initial increase of anti-apoptotic proteins observed on day 2<sup>nd</sup> and 4<sup>th</sup> of re-stimulation recedes on day 7<sup>th</sup> with enhanced production of pro-apoptotic proteins (Fig. 6C).



**Fig. 6**

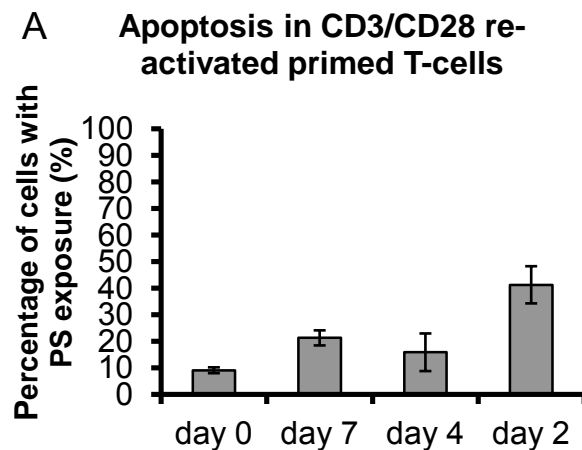
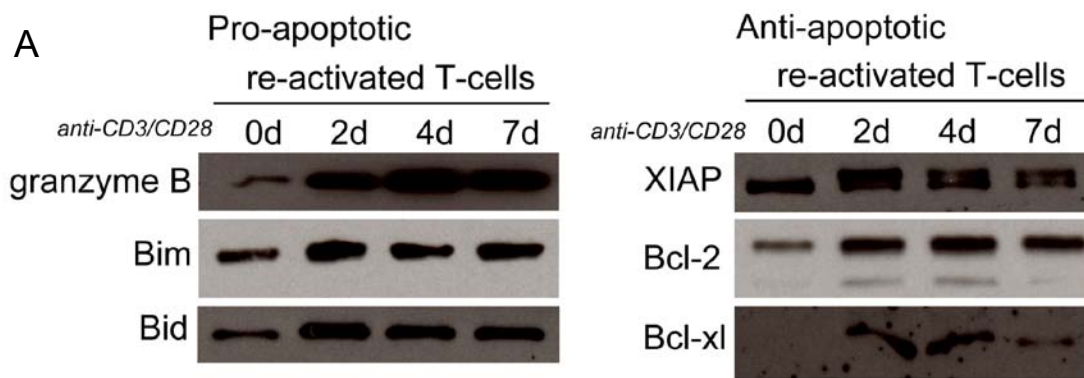


Fig. 6. AB. Evaluation of the number of cells with the apoptotic exposure of PS, as detected by Annexin V, showed increase in the apoptotic cells on days 2<sup>nd</sup> and 7<sup>th</sup> upon re-stimulation of primed CD4/CD8 cells (A). Increase of the amount of pro-apoptotic BID, Bim and Granzyme B (B) and decrease of anti-apoptotic Bcl-2, XIAP and Bcl-xl were noted on the day 7<sup>th</sup> upon re-stimulation, indicating



**Translational effect:** Ultimate goal of this project is to introduce the specific modification to the CEA specific designer T cells, which would counteract IL2 production shut down and population contraction of the designer T cells upon prolonged exposure to the antigen. Production of such cells will significantly prolong their systemic persistence and reactivity. Obtained results suggest that transcriptional factor Blimp-1 is one of the major players causing IL2 production shutdown. Expression of its repressor Bcl6 may counteract the process and will be explored in next experiments.

## KEY RESEARCH ACCOMPLISHMENTS

1. Development of procedures for improved gene modification
  - a. Use of retronectin to improve virus transduction
  - b. New vector supernatants with higher viral titer
  - c. Suitability of vector supernatant dilutions for clinical transductions
2. Development of procedures for improved expansions
  - a. High dose IL2
  - b. Prometheus application and approval to supply IL2 free of charge
  - c. Switch to heat-inactivated serum
  - d. Defined difference between patient and normal donor T cells for expansion
  - e. Adapting new technologies for dTc expansion (G-rex, wave bioreactor)
3. Established collaboration with Dr Waldman and obtained IL15 for Aim 2 studies
  - a. IL15 received
  - b. Initial experiments completed
4. Coordination with Dr Susan Love of the Army of Women foundation for patient recruitments.
  - a. Obtained approval
  - b. 70+ contacts
  - c. 7 enrollments in past year plus prior subject = 8 enrollments
  - d. 2 patients treated this year = 3 treatments
5. cGMP facility renovation and re-organization (underway)
6. Clinical
  - a. Phase Ia, previously completed with three subjects at the  $10^{10}$  dose level
  - b. Phase Ib: eight enrolled two treated
  - c. One treated with IL2 (but inevaluable for IL2), one treated without IL2, one awaiting dose production and treatment (-IL2 randomization)
  - d. Recruitment mechanisms installed to ramp up patient enrollments, including successful listing with Love/Avon Army of Women.
7. Pharmacokinetics
  - a. Standardization of PCR assay that is sensitive (to 0.01%)
  - b. Established comparison of PK with infusion versus engraftment
8. Tissue detection of dTc
  - a. Improved sensitivity and specificity
  - b. For the first time, now able to detect dTc in embedded, formalin-fixed tissue
9. Preclinical
  - a. Established Blimp-1 as candidate molecule mediating IL2 shutdown
  - b. Data supporting the functionality of human CAR chains in mouse T cells
  - c. Preliminary data on the benefit of dTc engraftment in animal model
10. Recruitment of Dr Lo to assist in completing evaluation of costimulatory constructs (previously indicated)
11. Recruitment of Dr Dubielecki to investigate mechanisms of shutdown of IL2 production (previously indicated)
12. Recruitment of Dr Guo to assume duties for high throughput analysis of costimulation molecules (new)

## REPORTABLE OUTCOMES

Publications (submitted or in preparation):

Michalczyk I, Sikorski AF, Kotula L, Junghans RP, Dubielecka PM. An intriguing connection between PKC $\theta$  and the cytoskeleton. *Cell Molec Life Sci*, Submitted, in 2<sup>nd</sup> review.

Bais A, Lo A, Borriello M, Yang W, Ma Q, Junghans RP. Use of degenerate oligonucleotides to suppress recombination-deletion of homologous elements in vectors for gene therapy. In preparation.

Gomes E, Ma Q, Selliah N, Junghans RP. Signal 3 modified chimeric antigen receptors increase IL2 production but do not prevent IL2 shutdown. In preparation.

Nadeem N, Junghans RP, Dubielecka P. IL2 signalling pathways. In preparation.

## CONCLUSION

Progress continues on the Aims of the proposal. The Phase Ib study initiated during the prior year and is set to accelerate recruitments after the renovation and re-organization of the gene therapy facility. Resources have been conserved to conduct these patient tests. The laboratory research continues to develop new agents along with a plan to understand IL2 needs of dTc.

## ATTACHMENTS

Resume:

Dr Xiuyang Guo (new researcher)

Dr Pranay Khare (new GMP Director)

Agreement:

Prometheus agrees to supply IL2 for clinical trial with high concentration IL2 for manufacturing

---

## REFERENCES

- <sup>1</sup> Emtage PCR, Lo ASY, Gomes EM, Liu DL, Gonzalo-Daganzo R, Junghans RP. 2<sup>nd</sup> generation anti-CEA designer T cells resist activation-induced cell death, proliferate on tumor contact, secrete cytokines and exhibit superior anti-tumor activity in vivo: a preclinical evaluation. Clin Cancer Res 2008;14:8112-22.
- <sup>2</sup> Lo ASY, Ma Q, Liu DL, Junghans RP. Anti-GD3 chimeric sFv-CD28/T cell receptor zeta designer T cells for treatment of metastatic melanoma and other neuroectodermal tumors. Clin Cancer Res 2010;16:2769-80.
- <sup>3</sup> Bais A, Borriello M, Yang W, Ma Q, Junghans RP. Development of retroviral vectors encoding anti-JC virus chimeric T cell receptors with degenerate codons. New England Immunology Conference, Woods Hole, October, 2009.

**PROMETHEUS LABORATORIES INC.****Amended and Restated Grant Agreement – Material Transfer**

June 13, 2011

Richard P. Junghans, Ph.D., M.D.  
Chief, Division of Surgical Research  
Associate Professor of Surgery and Medicine  
Director, Biotherapeutics Development Lab  
Roger Williams Medical Center  
825 Chalkstone Avenue  
Providence, Rhode Island 02908

Dear Dr. Junghans,

Roger Williams Medical Center and Prometheus Laboratories Inc. ("Prometheus") entered into a grant agreement on March 2, 2011 (the "Original Agreement") and now desire to amend and restate the Original Agreement to address certain changes to terms of the Original Agreement.

The Investigator Initiated Trial Review and Approval Committee ("ITTRAC") of Prometheus reviewed the Study synopsis of each of your proposed Study **"Phase Ia/Ib Trial of 2<sup>nd</sup> Generation Anti-CEA Designer T Cells in Metastatic Breast Cancer- Prometheus Protocol #IIT10PLK16"** on Exhibit A hereto ("the Study"), and determined that they each have scientific and medical merit. The ITTRAC of Prometheus is pleased to offer you (the "Recipient" or "you") a research grant in-kind (the Grant") in the form of a supply of Proleukin (aldesleukin) for use in the Study in the quantity and form as more fully described on Exhibit B hereto (the "Study Materials").

Our agreement to provide Study Materials is conditioned on the following:

1. All necessary regulatory approvals and Institutional Review Board ("IRB") approvals for the Study shall be obtained by Recipient, and Recipient will conduct the Study in accordance with all applicable laws, rules and regulations (including any applicable FDA and ICH guidelines), the Protocol and any applicable IRB requirements. Recipient will have obtained proper informed consent from each participant in the Study. Recipient will notify Prometheus promptly of any action by the Recipient's IRB altering its review or approval of the Study.
2. Recipient will notify Prometheus of, and provide Prometheus with a reasonable opportunity to review and comment upon, any proposed changes to the Protocol. Recipient shall also notify the IRB and all applicable regulatory bodies of any such change. However, Recipient shall have the full and final discretion over changes to the Protocol and Recipient will notify Prometheus when any changes have been finalized.
3. Recipient will provide Prometheus with copies of all reports submitted to FDA, and other government agencies, or an IRB that are related to the Study, as well as any correspondence with such entities related to the Study.



4. Recipient will provide Prometheus with copies of unpublished manuscripts, including abstracts, as well as a summary of any presentation relating to the Study at least 30 days in advance of submission to a journal or publication (for a manuscript or abstract) and 7 days in advance of any scientific meeting (for a presentation). At the request of Prometheus, Recipient will remove any Prometheus Confidential Information. No editorial rights or control over such publication or presentation by Prometheus is implied herein.
5. Recipient shall keep Prometheus informed of the status of the Study including, without limitation, Recipient shall a) meet with Prometheus, at times mutually agreed to by the parties to advise as to the status of the Study, and b) provide Prometheus, on a confidential basis, periodic reports on the status of the Study until the Study is completed, and with a final report within 90 days after Study completion.
6. Recipient shall permit Prometheus to conduct audits from time to time to verify compliance with the terms of this grant agreement. Any such audit shall be conducted by or on behalf of Prometheus at reasonable times as agreed to by the parties and with reasonable prior written notice. Recipient shall assist Prometheus in good faith and shall provide Prometheus with all such information necessary for Prometheus to confirm compliance with the terms of this research grant agreement.
7. Records relating to the Study will be retained for at least two years after the completion or earlier termination of the Study. Source documents, such as patient charts, will be retained for not less than five years.
8. The Recipient will use the Study Materials solely for the conduct of the Study.
9. The Recipient will not distribute the Study Materials to any third party.
10. The Recipient shall return any unused supplies of Study Materials to Prometheus at the completion or earlier termination of the Study.
11. PROMETHEUS MAKES NO REPRESENTATIONS AND EXTENDS NO WARRANTIES OF ANY KIND, EITHER EXPRESSED OR IMPLIED WITH RESPECT TO THE STUDY MATERIAL INCLUDING WITHOUT LIMITATION, WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, OR THAT THE USE OF THE MATERIAL WILL NOT INFRINGE ANY PATENT, COPYRIGHT, TRADEMARK, OR OTHER PROPRIETARY RIGHTS.
12. The Recipient shall acquire no intellectual or other proprietary rights of any kind with respect to the Study Materials or other products or materials provided by Prometheus. Recipient will notify Prometheus immediately upon filing a patent application on any invention made while using the Study Materials furnished to Recipient under this Agreement. Nothing herein shall be construed as granting any assignment or license under any patent, trademark, trade secret or copyright of Prometheus.
13. You will maintain in confidence, and only use for the Study, any confidential or proprietary information we provide or disclose to you in connection with the Study.
14. Unless prohibited by law, Recipient agrees to hold harmless and indemnify Prometheus against any claims or lawsuits for damages that arise out of the Study. Prometheus agrees to notify you promptly of any such claim and to assist you and your representatives in the investigation and defense of any lawsuit and/or claim for which indemnification is provided hereunder. You shall not settle any claim for Prometheus without Prometheus' prior written consent, which shall not be unreasonably withheld.

15. The Grant will only be used for the Study and any funds that are not used for the Study will be refunded to Prometheus upon completion or earlier termination of the Study. If the Study is not conducted in accordance with the Protocol, you will return the Grant to Prometheus promptly upon Prometheus request.

16. Prior to receiving any payment under this grant agreement, the Recipient shall provide Prometheus with a sufficiently detailed invoice for each requested payment.

17. The Grant imposes no obligation, express or implied, for you to purchase, prescribe, provide favorable formulary status for, or otherwise support Prometheus products.

The foregoing represents the entire grant agreement between us regarding the Study, and there are no further commitments, obligations or understandings of any nature regarding the Study. This grant agreement will be effective on the date of the last signature below, and will expire, unless earlier terminated in accordance with terms hereof, upon the completion of the Study and our payment of all amounts required hereunder. Prometheus shall have the right to terminate this grant agreement at anytime during the grant agreement with written notice to Recipient. The provisions of Sections 7, 8, 9, 10 and 13 shall survive the expiration or earlier termination of this grant agreement. This grant agreement may be amended with the mutual written consent of the parties.

If the above terms are acceptable to you, please sign both copies of this letter agreement in the space provided below and return one original to my attention.

Sincerely,

PROMETHEUS LABORATORIES INC.

By:

Name: JOSEPH M. LIMBER  
Title: PRESIDENT & CEO

*Jim*  
8/31/11

Accepted and agreed to:

*Karen Geremia*

By: Karen Geremia, Director Research Department

Date:

7-29-11

Acknowledged and agreed to by:

*[Signature]*

Richard P. Junghans, Ph.D., M.D.

Approved By  
Prometheus  
Finance Dept.

*[Signature]*

**Exhibit A**

**See Attached Protocols:**

**"Phase Ia/Ib Trial of 2<sup>nd</sup> Generation Anti-CEA Designer T Cells in Metastatic Breast Cancer-  
Prometheus Protocol #IIT10PLK16"**



**Exhibit B**

Number of patients to treat for the entire study: 12

Number of patients already enrolled treated: 2 (Non evaluable)

Number of patients yet to treat under March 15, 2010 protocol: 12

**Phase Ib - IL-2**

Per pt: 7 vials for manufacturing x 6 pts = 42 vials

**Phase Ib + IL-2**

Per pt: 7 vials for manufacturing x 6 pts = 42 vials, and 12 vials for each pt receiving IL2  
x 6 pts= 72 vials

156 vials for Phase Ib

40 vials overage if any patients do not complete treatment and have to be replaced.

---

196 vials to complete the study

**PRANAY D. KHARE, PH.D.**

**SUMMARY:**

- Leader with translated 14+ years of biologic drug discovery including viral and non-viral based Immuno-Gene and Gene Therapy products for Oncology, Ophthalmology and Immunology field. Multiple patents on novel eukaryotic display technology.
- Process Development: Designer cell lines, viral and non-viral vectors process development studies, cGMP production and risk assessment of novel therapeutic in cancer research.
- Clinical Experience: 5+ years to oversee clinical studies with cross functional teams (Clinical, Regulatory, Biostatistics, Biomarkers, Translational Medicine and Clinical Lab results, etc.). Developed novel clinical assay based on T, B and NK cells.
- Regulatory Experience: 6+ years with development of SOPs, Batch Records, CMC, IRB, FDA reports. Communication with FDA, NCI, CLIA and New York State Department of Health.
- Managerial Experience: 5+ years in small and Fortune 500 Biotech companies. Advise and organize multi-divisional teams, develop timeline and execution strategies.
- Grants and Funding: Recipient of several funding from Federal and prestigious international organizations in academic and corporate work environment.
- Communication Skills: Correspond, write, review manuscripts, white papers, product's marketing and sales materials; develop new business strategies, technologies and proposals.

**EXPERIENCE:**

10/11 to  
Present

**COSMOS BIOSYSTEMS, INC., Colfax, WI**

Chief Scientific Director

- Developing novel and effective strategies for functional assays for chronic neurological and immunological diseases.
- Develop DNA diagnostic strategies to utilize company's platform technology for human pathogens (bacterial, viral and fungal), environmental and agriculture pathogens.
- Strategic planning on scientific communication, business and organizational development, marketing and medical affairs.

01/09 to  
08/11

**NEUROSCIENCE, INC., Osceola, WI**

Scientific Director

- Primary role is to vision and direct company's overall research and education particularly focused on Neuroimmunology. Initiate and finalize clinical collaborative studies for inflammatory and CNS diseases.
- Develop IRBs and clinical protocols (including trial design, population selection, assessment of efficacy, safety, PK, PD study evaluations, support QA/ QC group).
- Design, validate, prepare, submit and receive approval from CLIA for several immunoassays (cell based [T, B, NK cells], FACS, Cytokine and ELISA) for immune and cancer diseases.
- Apply cutting edge technologies to identify the role of biomarkers (multiplex, -omics) in human inflammatory diseases (tumor, infectious) using cutting edge in vitro diagnostic (IVD) tools.
- Significantly enhance product pipeline and robust accelerate in revenue growth (millions of US dollars) from novel clinical tests and products.
- Develop and execute publications strategies, successful grant submission and review and deliver presentations (paper, PowerPoint slides, multimedia, webinar, and seminar) at national and international scientific and clinical meetings.

- Develop relationship and scientific communication with Key Opinion Leaders, physicians, customers, sales and marketing team.
- 2008 to 2009     **BIO-PHARMACEUTICAL DEVELOPMENT PROGRAM, SAIC INC.,** Frederick, MD  
Senior Development Scientist
- Supervise the process development of different gene therapy product including viral (adeno, retro, lenti, adeno-associated) vectors, oncolytic viruses and cell based products for clinical use and their GLP and cGMP grade production in highly regulated environment.
  - Translation of different novel adenoviral, herpes-simplex virus, poliovirus and other viral vectors for anti-cancer strategy using process development strategies.
  - Review and supervised the feasibility studies for the manufacturing of viral vectors and viruses using highly regulated SOPs.
  - Provide communication support with government organization (FDA, NIH, NCI).
- 2007 to 2008     **GENE THERAPY PROGRAM, U OF PENNSYLVANIA,** Philadelphia, PA  
Associate Director-Quality Control
- Direct and supervise the development and characterization of novel viral vectors for targeted therapy and vaccine strategies against several human diseases.
  - Developing novel assays to determine the quality, potency and stability of Adenovirus, Adeno-associated virus, Lentivirus, and Retrovirus. Developed novel quantitative real-time PCR based assay to determine the genome titer, TCID<sub>50</sub>.
  - Supervised four research technicians and directs the goals of Quality Control core facility.
- 2006 to 2007     **NEOTROPIX INC.,** Malvern, PA  
Senior Scientist III
- Develop and genetic engineer oncolytic virus to determine their therapeutic efficacy.
  - Determine the genomic characterization (full-length nucleotide sequencing) of company's product an Oncolytic virus (Seneca Valley virus) for our Phase I clinical trial.
  - Established and transfer assays to clinical department: Determined the nature of our virus after virotherapy and gene therapy in Phase I clinical trial and virus clearance.
  - Vision, developed, established and validate a very relevant and possible biomarker to support drug development and therapeutic.
  - Originator, author and reviewer of several clinical reports submitted to FDA in support of Phase I clinical trial.
- 2000 to 2006     **MAYO CLINIC, MOLECULAR MEDICINE PROGRAM,** Rochester, MN  
Scientist
- Developed different Feline virus based Lentiviral vectors and Avian and Murine based retroviral vectors for target validation in gene therapy settings.
  - Performed PK and PD studies in pre-clinical studies and examined therapeutic efficacy of viral products in different anti-angiogenesis, anti-tumor (breast, prostate and pancreatic model) and eye disease model in small and large laboratory animals (including non-human primates).
  - Patented a novel polypeptide display technology by displaying variety of peptides, proteins, protein scaffolds, antibodies and T cell receptor on eukaryotic virus.
  - Construct and genetically modified peptides, protein scaffolds, T cell receptor and antibody display libraries and developed assays to screen them in vitro and in vivo.
  - Published several manuscripts, write and received several funding in support of my cutting edge research.

- 1997 to 2000 **FUKUOKA UNIVERSITY**, Fukuoka, Japan  
Visiting Fellow and Postdoctoral Fellow
- Solely visions, think, developed and established gene (viral vectors) and immune-gene (T-cell) therapy products for gastric and colon cancer.
  - Developed tumor targeted Murine retroviral vector and examined their therapeutic efficacy in several in vivo tumor xenograft in SCID and Nude mice including gastric and colorectal tumors.
  - Creator and author of several peer-reviewed publications and grants.
- 1993 to 1997 **KING GEORGE MEDICAL COLLEGE**, Lucknow, India  
PhD Research Fellow
- Identify, purify and characterized of a novel receptor for cytotoxin from murine T cells and macrophages in dengue virus disease in mice.
  - Established and documented different proteins and their receptors purification using HPLC, LPLC and affinity purification methods.
  - Develop dengue virus vaccine strategy model using cytotoxin protein.
  - Accomplished several grants, presentations and publications in peer-reviewed journals.

## **EDUCATION:**

- 1998 **SSJM Kanpur University**, Kanpur, India  
Ph.D. Life sciences
- 1993 **Inst. of Life Science, Kanpur University**, Kanpur, India  
M.S. Life sciences
- 1989 **Kanpur University**, Kanpur, India  
B.S. Biology Group

### ***Certifications:***

- Quality Control and Quality Assurance, University of Maryland at BC, MD (2008)
- Management Development Program, SAIC-Frederick, Frederick, MD (2009)

### ***Awards and Fellowships (since year 2000):***

- Executive and Professional Directors in Strathmore's Who's Who (2010)
- Executives and Professionals in Madison Who's Who (2007 to 2008)
- Member Continental Who's Who (2007 to 2008)
- Exceptional Scientist in biological and engineering field in Madison Who's Who (2006)
- Exceptional Scientist in biological field by Marquis Who's Who (2005)
- Member Grant Review Committee, Susan G. Komen Breast Cancer Foundation, USA (2004 to 2005)
- Breast Cancer Research Career Mentoring Award from Susan G. Komen Breast Cancer Foundation, USA
- Travel grant for Third Annual Future of Breast Cancer Congress, Bermuda
- Breast Cancer Research Career Mentoring Award from The Susan G. Komen Breast Cancer Foundation, USA (2004)
- Breast Cancer Research Co-Mentoring Program Award from The Susan G. Komen Breast Cancer Foundation, USA (2003)
- Best Poster Award, Mayo Clinic, Rochester, MN, USA (2001)

***Funding Supports:***

- Immune Tolerance Test (ITT)-Cytokine Platform Project under Qualifying Therapeutic Discovery Project Grant US Government, IRS, USA.
- Process development Studies for MVA Production. NCI, NIH, USA.
- Selection of human breast cancer specific peptides from glycosylated Avian Leukosis
- Virus displayed peptide library. Susan G. Komen Breast Cancer Foundation, USA.
- Selection of multiple myeloma and angiogenesis specific-targeted peptides from glycosylated peptide libraries displayed on avian leukosis virus. Leukemia Research Foundation, USA.
- Targeted Retroviral Vector for Gastric Cancer. Nippon Health Science Foundation, Japan.
- Characterization of receptor for dengue virus-induced macrophage cytotoxin. Council of Scientific and Industrial Research, India.
- Determine the cell death mechanism by cytotoxin. Lady Tata Memorial Trust, India.

***Intellectual Property:***

- US Patent 7132237: Avian Leukosis Viruses and Polypeptide display. Mark J. Federspiel, Stephen J. Russell and Pranay D. Khare (Nov 2006).
- US Patent 7449322: Avian Leukosis Viruses and Polypeptide display. Mark J. Federspiel, Stephen J. Russell and Pranay D. Khare (Nov 2008).
- US Patent 7625742: Avian Leukosis Viruses and Polypeptide display. Mark J. Federspiel, Stephen J. Russell and Pranay D. Khare (Dec 2009).

***Publications in Peer-Reviewed Journals (since year 2000):***

- Khare, P.D., Loewen, N., Teo W. L., Barraza, R, Saenz, D.T., Johnson, D.H., and Poeschla, E.M. Durable, safe, multi-gene lentiviral vector expression in feline trabecular meshwork. *Molecular Therapy*. 16(1), 97 – 106, 2008.
- Khare, P.D. Loewen, N, and Poeschla, E. M. Suppression of human pancreatic cancer growth by lentiviral vector-mediated gene transfer of pigment epithelial derived growth factor (PEDF). *Molecular Therapy*, 13(S1): S313 – S314, 2006.
- Fujimura, S., Arakawa, F., Suzumiya, J., Yamada, Y., Liao, S., Khare, P. D., Kuroki, Ma. and Ono, J. Growth arrest and apoptosis in an adult T cell leukemia cell line following IL-2 deprivation. *International Journal of Oncology*. 25 (2): 437-443, 2004.
- Khare, P. D., Russell, S. J. and Federspiel, M. J. Avian leukosis virus is a versatile platform for polypeptide display. *Virology*. 315(2): 303-312, 2003.
- Khare, P. D., Rosales, A. G., Bailey, K. R., Russell, S. J. and Federspiel, M. J. Epitope selection from an uncensored peptide library displayed on avian leukosis virus. *Virology*. 315(2): 313-321, 2003.
- Khare, P. D., Khare, M., Tandon R and Chaturvedi, U. C. Identification, purification and characterization of a receptor for dengue virus-induced macrophage cytotoxin (CF2) from murine T cells. *FEMS Immunology and Medical Microbiology*, 38(1) 35-43. 2003. (Corresponding author)
- Khare P. D., Liao, S., Hirose, Y., Kuroki, Mo, Fujimura, S., Yamauchi, Y., Uchida, H., and Kuroki, Ma. Tumor growth suppression by a retroviral vector displaying scFv antibody to CEA and carrying the iNOS gene. *Anticancer Research*. 22, 2443-46, 2002.
- Khare P. D., Shao-Xi L., Kuroki, Mo, Hirose, H, Arakawa, F., Nakamura, K., Tomita, Y. and Kuroki, Ma. Specifically targeted killing of carcinoembryonic antigen-producing (CEA)-expressing cancer cells by a retroviral vector displaying single chain variable fragmented antibody to CEA and carrying the gene for inducible nitric oxide (iNOS). *Cancer Research*, 61, 370-375, 2001.
- Liao, S., Khare, P. D., Kuroki, Mo., Arakawa, F., Tomita, Y., Kuroki, Ma. Targeting of LAK activity to CEA-expressing tumor cells with a recombinant fusion protein of IL-2 and anti-CEA scFv antibody. *Anticancer Research*. 21: 1673-1680, 2001.

- Beppu R., Nakamura K., Uchida H., Kuroki, Mo, Khare P. D., Yamauchi, Y., Yamashita, Y., Shirakusa T. and Kuroki Ma. Soluble thrombospondin-1 suppresses T cell proliferation and enhances IL-10 secretion by antigen presenting cells stimulated with phytohemagglutinin. Immunological Investigation. 30(2): 143-156, 2001.
- Kuroki, Ma. Arakawa, F., Khare, P. D., Kuroki, M., Liao, S., Matsumoto, H., Abe, H., and Imakiire, T. Specific targeting strategies of cancer gene therapy using a single-chain variable fragment (scFv) with a high affinity for CEA. Anticancer Research. 20: 4067-4072, 2000.
- Khare P. D., Liao, S. Kuroki Mo., Arakawa F., and Kuroki Ma. Specific gene therapy of CEA-expressing tumor cells using reconstructed retrovirus. Proceedings of 2nd Congress of the Federation of Immunological Societies of Asia-Oceania (FIMSA), 51-56. 2000.  
Tomita, Y., Arakawa, F., Hirose, Y, Liao, S., Khare, P. D., Kuroki, Mo., Yamamoto, T., Ariyoshi, A., and Kuroki, Ma. Carcinoma-associated antigens MK-1 and CEA in urological cancers. Anticancer Research. 20: 793-798, 2000.
- Khare, P. D. and Kuroki Ma. Specific cancer gene therapy. Research, 4, 8-9, 2000.

***Other Publications and Abstract Presentations:***

- Oral Presentation: 25 oral presentations, including Travel grants and New investigator awards.
- Poster Presentation: 41 poster presentations; including four Travel Grants.
- Seminars and Webinar Presentations: Key presenter on company's overall scientific, integrative and functional medicine approach to address variety of chronic neurological and neuropsychiatric diseases in national and regional meetings.
- Invited Presentations: More than 30 presentations in US, Japan, India and Australia.

***Other Publications:***

- [https://www.neurorelief.com/uploads/content\\_files/MELISA%20Paper-Poster.pdf](https://www.neurorelief.com/uploads/content_files/MELISA%20Paper-Poster.pdf)
- [https://www.neurorelief.com/uploads/content\\_files/LymePoster.pdf](https://www.neurorelief.com/uploads/content_files/LymePoster.pdf)
- [https://www.neurorelief.com/uploads/content\\_files/Cytokine%20Physiology%20and%20Clinical%20Relevance.pdf](https://www.neurorelief.com/uploads/content_files/Cytokine%20Physiology%20and%20Clinical%20Relevance.pdf)
- <https://www.neurorelief.com/index.php?p=cms&cid=87&pid=18>

## Curriculum Vitae

### Xiuyang Guo

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### Education

**Ph.D. in Biochemistry and Molecular Biology**, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, September 2000 - March 2005, under the supervision of Prof. Changde Lu.

Title of thesis: Study on Genetical Manipulation Technique and Its Application in Silkworm, *Bombyx mori*.

As a doctoral student, I held the following award: The excellent student scholarship (Biochemistry and Cell Biology Institute, Chinese Academy of Sciences, 2003).

**M.S.** In the area of molecular biology of silkworm, Department of Sericulture, Silkworm and Silk College, Southwest Agricultural University, Chongqing, China, September 1997 - July 2000, under the supervision of Prof. Zeyang Zhou.

Title of thesis: Sperm-mediated Genetic Transformation of Silkworm.

As a master's student, I held the following award: Honored as Excellent Graduate (Southwest Agriculture University, 2000).

**B.Sc.** Major in Sericulture, Department of Sericulture, Silkworm and Silk College, Southwest Agricultural University, Chongqing, China, September 1993-July 1997.

As an undergraduate student, I held the following award: The excellent student scholarship (Southwest Agricultural University, 1996 - 1997).

## Research Experience

**Associate Research Scientist** In the Laboratory of Dr. Kavita Dhodapkar, Department of Pediatrics, Yale University, New Haven, CT, USA, June 2011-present.

### Projects:

- **Role of Cathepsin B and Inflammasome Adaptor ASC In Antigen Presenting Function Of Human Dendritic Cells.**

In this project I found DC maturation stimuli lead to caspase-1 activation in human monocyte-derived DCs. RNAi mediated inhibition of the inflammasome component ASC shows marked inhibition of the capacity of lipopolysaccharide (LPS)-matured DCs to stimulate antigen specific T cells. RNAi mediated inhibition of Cathepsin B (CatB) also similarly inhibits the capacity of human DCs to stimulate immunity. The defective ability of ASC or CatB deficient DCs to stimulate T cells is not restored following maturation with exogenous inflammatory cytokines, suggesting that it is independent of the capacity of inflammasomes to process these cytokines. Gene expression profiles of ASC or CatB deficient human DCs show marked overlap with down regulation of several genes implicated in DC function, but differ from those recently shown to be altered in murine ASC deficient DCs. These data demonstrate an important role for ASC and CatB in regulating function of human DCs and suggest that the effects of these proteins on gene expression may also contribute to their roles in the immune system.

- **Immunopathogenesis of Autoimmune Disease Idiopathic Thrombocytopenic Purpura (ITP).**

In this project I used blood samples from ITP patients in a clinical trial before and after IVIG treatment, and healthy donors for control. After comparing their monocyte gene expression profiles, some interesting activated pathways such as interferon alpha signaling pathway specifically in ITP patients were identified. The ongoing study includes characterization of the mechanisms that cause this activation, such as the possible involvement of some specific microparticles and immune complex from the patients. I am also trying to identify the effect components in IVIG such as sialylated IgG that functions to temporally relieve the disease.

- **Immune system modulation by anti PD1 treatment in small cell lung cancer patients.**

In this ongoing project blood samples from different cycles of continuous anti PD1 treated small cell lung cancer patients in a clinical trial were collected. Monocytes gene expression profile were compared between patients with/without response in tumor regression upon anti PD1 treatment. T cell phenotype (by LSRII FACS) and function (upon in vitro stimulation) will be compared.

**Postdoctoral Associate** In the Laboratory of Dr. Kavita Dhodapkar, Department of Pediatrics, Yale University, New Haven, CT, USA, June 2009- May 2011.

### Projects:

- **Inflammasome in Human Dendritic Cell Immune Function.**

(see above.)

- **Immunopathogenesis of Idiopathic Thrombocytopenic Purpura (ITP).**

(see above.)



**Postdoctoral Associate** In the Laboratory of Dr. Ruth Montgomery, Department of Internal Medicine, Yale University, New Haven, CT, USA, June 2007- May 2009.

Projects:

- **Mechanism of Vector Saliva Inhibition of Innate Immunity.**

In this project two Ixodes proteins that could be used as vaccines for prevention of pathogen transmission from ticks to animals and human were identified. Using saliva harvested from ticks with reduced levels of the two proteins through targeted RNA interference knockdown, as well as purified recombinant proteins, I showed the effects of these proteins on downregulation of PMN integrins and inhibition of the production of superoxide by PMN in vitro. Mice immunized with the two proteins had increased numbers of PMN at the site of tick attachment and a lower spirochete burden in the skin and joints compared to control-immunized animals.

- **Molecular Mechanism of Neutrophil Function and Aging.**

In this project I examined the expression and function of TLRs on PMN from younger (21-30 years) and older (>65 years) adults and found that PMN from the elderly expressed lower surface levels of TLR1 and 4. I found that there is significantly lower increase of surface Mac-1 on neutrophil of the elderly upon stimulation by LPS or TNF- $\alpha$  despite the fact that they have a higher basal level. Also, there are higher basal levels of CCL4 and IFN $\gamma$  in neutrophil of the elderly. However, significantly lower increases in Akt and Stat3 activation upon TLR1/2, TLR4 ligands or TNF- $\alpha$  stimulation in neutrophils of the elderly were observed. These results demonstrate that although in a slightly activated basal status, the TLR expression and function for neutrophil of the elderly are declining, which may contribute to the enhanced susceptibility to infections and poor response to vaccines in aging.

**Postdoctoral fellow** In the Laboratory of Prof. Reuben Kaufman, Biological Sciences Department of University of Alberta, Edmonton, Canada, May 2005 - May 2007.

Projects:

- **Toward Developing a Vaccine to Inhibit Tick Feeding.**

The lab identified two proteins from tick *vas deferens* that was shown to be important for female tick engorgement and reproductive maturation. In this project I tried expression/purification of these two proteins in insect cell expression system for testing their effectiveness in inhibiting tick feeding by using them as vaccines to immunize the host.

- **Functional Screening of Fed Induced Tick Male Gonad Genes.**

In this project I used RNA interference technology to investigate the consequences of blocking the function of fed induced tick male gonad genes. Attenuation of the expression of two of these genes correlated with deformities in the testis and abnormalities in spermiogenesis. Furthermore, most females fed in the company of these males did not engorge properly and laid many fewer eggs, most of which were infertile.

## **Hands-on Research Techniques**

Immunology experiments, mouse model, and biomedical study general technologies, such as DNA/RNA/Protein extraction and analysis, tissue culture, western blot, ELISA, immunofluorescence, FACS (LSR II/ Calibur), Luminex, real time PCR, protein expression and purification etc.

## **Professional Membership**

- Member, The American Association of Immunologists, 2011-Present.

## **Manuscript in Progress**

**Xiuyang Guo**, Kavita Dhodapkar. Central and overlapping role of cathepsin B and inflammasome adaptor ASC in antigen presenting function of human dendritic cells. Paper in submission, 2012

## **Peer-reviewed Publications**

1. **Xiuyang Guo**, Carmen J. Booth, Michael A. Paley, Xiaomei Wang, Kathleen DePonte, Erol Fikrig, Sukanya Narasimhan, and Ruth R. Montgomery. Inhibition of neutrophil function by two tick salivary proteins. *Infect Immun*, 2009 June; 77(6):2320–2329
2. **Xiuyang Guo** and Reuben Kaufman. Identification of two genes essential for sperm development in the male tick *Amblyomma hebraeum* Koch (Acari: Ixodidae). *Insect Biochem Mol Biol*. 2008 Jul; 38 (7): 721-729
3. **Xiuyang Guo**, Yi Zhang, Xue Zhang, Shengpeng Wang, Changde Lu. Recognition of Signal Peptide by Protein Translocation Machinery in Middle Silk Gland of Silkworm *Bombyx mori*. *Acta Biochimica et Biophysica Sinica*. 2008 Jan; 40 (1):38-46
4. **Xiuyang Guo**, Shengpeng Wang, Tingqing Guo, and Changde Lu (2005). Silkworm (*Bombyx mori*.) Silk gland Specific Secretory Expression of EGFP with rAcMNPV System. *Arch Virol*. 2005 Jun; 150 (6): 1151-1160

5. **Xiuyang Guo**, Liang Dong, Shengpeng Wang, Tingqing Guo, Jianyang Wang, and Changde Lu (2004). Introduction of Foreign Genes into Silkworm Eggs by Electroporation and Its Application in transgenic Vector Test. *Acta Biochimica et Biophysica Sinica*, 36 (5): 323-330
6. **Xiuyang Guo**, Zeyang Zhou, Lichun Feng, Lin Wang , Cheng Lu , Zhonghuai Xiang (2001). Sperm-mediated Gene Transformation of Silkworm. *Prog. Biochem. Biophys.*, 28 (3): 423-425
7. **Xiuyang Guo**, Zeyang Zhou (1999). The Status quo of Transgenic silkworm study. *Sericulture Communications*, 19 (4): 14-18
8. Alexander Smith, **Xiuyang Guo**, José de la Fuente, Victoria Naranjo, Katherine M Kocan and W Reuben Kaufman. The impact of RNA interference of the subolesin and voraxin genes in male *Amblyomma hebraeum* (Acari: Ixodidae) on female engorgement and oviposition. *Exp Appl Acarol.* 2009 Jan; 47(1):71-86
9. Shengpeng Wang, Tingqing Guo, **Xiuyang Guo**, Juntong Huang, Changde Lu. In vivo analysis of fibroin heavy chain signal peptide of silkworm *Bombyx mori* using recombinant baculovirus as vector. *Biochem Biophys Res Commun.* 2006 Mar 24; 341 (4): 1203-1210
10. Yan Liu, Lian Yu, **Xiuyang Guo**, Tingqing Guo, Shengpeng Wang, Changde Lu. Analysis of Tissue-specific Region in Sericin 1 Gene Promoter of *Bombyx mori*. *Biochem Biophys Res Commun.* 2006 Mar 31; 342 (1): 273-279
11. Tingqing Guo, Jianyang Wang, Shengpeng Wang, **Xiuyang Guo** , Kewei Huang, Juntong Huang and Changde Lu. Loss of posterior silk gland transcription specificity of fibroin light chain promoter due to absence of 41 bp sequence containing possible inhibitor binding sites. *Acta Biochim Biophys Sin (Shanghai).* 2005 Dec; 37 (12): 819-825
12. Tingqing Guo, Shengpeng Wang, **Xiuyang Guo** and Changde Lu. Productive infection of *Autographa californica* nucleopolyhedrovirus in silkworm *Bombyx mori* strain Haoyue due to the absence of a host antiviral factor. *Virology.* 2005 Oct 25; 341 (2): 231-237
13. Yuanyuan Li, Lei Wang, Tingqing Guo, **Xiuyang Guo**, Peijun Yan, Ying Chen, Changde Lu. p53 protein activates the transcription of human proliferating cell nuclear

antigen in response to 4-nitroquinoline N-oxide treatment. *Int J Biochem Cell Biol.* 2005 Feb; 37 (2): 416-426

14. Tingqing Guo, Jianyang Wang, Shengpeng Wang, **Xiuyang Guo**, Kewei Huang, Junting Huang and Changde Lu. Transient in vivo gene delivery to the silkworm *Bombyx mori* by EGT-null recombinant AcNPV using EGFP as a reporter. *Arch Virol.* 2005 Jan; 150 (1): 93-105